PASSIVE LEG MOVEMENT-INDUCED VASODILATION: IMPACT OF AGING IN WOMEN, PHYSICAL ACTIVITY, AND OXIDATIVE STRESS

by

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STATEMENT OF DISSERTATION APPROVAL

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has been approved by the following supervisory committee members:

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ABSTRACT

The overall objective of this dissertation was to further elucidate alterations in vascular function with age in healthy humans by utilizing the passive leg movement (PLM) model, with and without a posture-induced increase in femoral perfusion pressure (FPP), to assess nitric oxide (NO) bioavailability. In the first study, we examined the central and peripheral hemodynamic responses to PLM in young and old women. We observed a two- to three-fold attenuation in the leg blood flow (LBF) and leg vascular conductance (LVC) responses to PLM in the old women compared to the young women. These findings indicate that, similar to men, aging significantly attenuates vascular function in healthy women. The second study investigated the possible impact of elevated physical activity and fitness on the attenuated vascular function with age. Utilizing PLM, we documented that older individuals who remained physically active displayed improved vascular function compared to their sedentary age-matched counterparts, although not achieving the same vascular function as young sedentary individuals. In this study, we took these findings one step further by examining elite endurance trained older subjects and found that while they too demonstrated improved vascular function compared to sedentary age-matched subjects, vascular function failed to be restored to the level observed in the young, suggesting that there may be a ceiling effect whereby increasing physical activity yields diminishing returns in terms of vascular health. The third study sought to determine the role that oxidative stress may play in the age-related

attenuation in vascular function by administering both a placebo and an oral antioxidant cocktail (AOC) in a single-blind cross-over design. In the placebo condition, the old subjects once again had an attenuated vasodilatory response to PLM. Although displaying greater inflammation than the young, the old did not clearly exhibit elevated markers of oxidative stress or decreased markers of antioxidant defenses. The AOC significantly improved antioxidant status and decreased lipid peroxidation in both the young and old. However, the AOC had no effect on the vasodilatory response to PLM in either group. Therefore, vascular dysfunction persisted in the older adults despite an AOC-induced increase in antioxidant status and attenuated oxidative stress, questioning the role of redox balance in the apparent age-related decrease in PLM-induced vasodilation. Collectively, this research has provided significant insight into the impact of aging in women, physical activity, and oxidative stress on alterations in NO-mediated vascular function across the lifespan.

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First and foremost, I dedicate this doctoral dissertation to my parents, Willem and Cheryl Groot. They have made countless sacrifices, many of which I will probably never even know, to ensure that I was successful in my academic endeavors. I further dedicate this dissertation to my second father and mentor, Doug Jepperson, for helping me learn mental toughness, without which I would not have made it this far.

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CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD) is the number one cause of morbidity and mortality in the United States and other developed nations (10). Each day, more than 2,150 Americans die of CVD, accounting for 1 out of every 3 deaths per year. Aging is a major risk factor for CVD. Indeed, the average annual rate of first cardiovascular events increases 25-fold between the ages of 40 and 90 years. The impact of aging on CVD progression depends, at least to some extent, on the health and function of the vascular endothelium and, specifically, bioavailability of the endothelium-derived vasodilator nitric oxide (NO) (21, 23). NO has been demonstrated to be antiatherogenic (25), and NO bioavailability is lower in populations with hypertension (34), hyperlipidemia (3), diabetes (41), and heart failure (16), as well as with age (1, 7, 44).

In the vasculature, NO is an endothelium-derived vasodilator that is synthesized by endothelial nitric oxide synthase (eNOS) in response to a variety of stimuli, such as increased wall shear stress, cellular deformation, and estrogen (Figure 1.1). Activation of eNOS by the calcium-calmodulin complex allows oxygen, NADPH, and $_{L}$-arginine to be converted to water, $NADP^{+}$, $_{L}$-citrulline, and NO. Importantly, the production of NO can be up regulated or down regulated by the phosphorylation of eNOS at various stimulatory or inhibitory phosphorylation sites. NO then diffuses into the adjacent vascular smooth muscle cells and, through a cyclic GMP-dependent pathway, leads to a fall in intramyocellular calcium levels, smooth muscle cell relaxation, and vasodilation. Coupled with its antiatherogenic properties, this makes NO one of the most important molecules in the maintenance of vascular function and health. Although it is recognized that NO bioavailability declines with advancing age, currently, the mechanisms responsible for this fall in NO bioavailability and subsequent attenuation in vascular

function with age are not fully understood. However, evidence suggests that two possible mechanisms, the adoption of a sedentary lifestyle (39) and increased vascular oxidative stress (51), may play a role. Additionally, the literature regarding vascular function in women is far less extensive than that focused upon men; therefore, alterations in NO bioavailability with age in the female population remains poorly characterized.

Premenopausal women have a reduced risk of CVD compared to men and postmenopausal women. This observation has led to the belief that young women possess some sort of inherent cardioprotection. Estrogen, which declines drastically from pre- to postmenopause, has been documented to have powerful vascular effects, and may help explain the lower CVD risk in young women. Indeed, postmenopausal women display significantly attenuated endothelium-dependent vasodilation compared to premenopausal women (2, 14). This attenuated vascular function in postmenopausal women can be reversed by estrogen replacement therapy (14, 20), implicating diminished estrogen content as the primary cause of vascular dysfunction in old women. Furthermore, in mice, ovariectomy to simulate the postmenopausal decrease in estrogen significantly attenuates endothelium-dependent vasodilation, which can be restored by exogenous estrogen supplementation (24, 32).

The mechanisms by which estrogen provokes this endothelium-dependent vasodilation include increased cyclooxygenase production of vasodilating prostaglandins, the elevated release of endothelium derived hyperpolarizing factor, the inhibition of NADPH oxidase and subsequently the attenuation of superoxide production, and the inhibition of calcium influx into vascular smooth muscle cells (32, 38). However, given the significant role of NO in cardioprotection, perhaps the most important estrogen-

mediated mechanisms in the vasculature involve the augmentation of NO production by the up regulation of eNOS transcription and increased eNOS phosphorylation (26, 32, 38). Despite the clear importance of investigating alterations in vascular function with age in women, and the prevalence of peripheral vascular dysfunction in the lower limbs, to our knowledge, there has yet to be a single study examining NO-mediated vasodilation in the lower limbs of pre- and postmenopausal women.

Individual levels of physical activity and fitness may also impact CVD progression with age. Atherosclerotic lesions tend to form at arterial bifurcations (i.e., branch points in the arterial tree), which are characterized by low mean blood velocity, high retrograde shear, and high oscillatory shear forces on the luminal surface of the arterial wall (22, 33, 49). This proatherogenic shear pattern is augmented with advancing age (33). During dynamic exercise, anterograde blood flow is profoundly increased to active skeletal muscle, leading to high mean blood velocity, a reduction in oscillatory shear, and an overall antiatherogenic blood flow profile (19, 42). Additionally, the acute stress placed on the vasculature during exercise increases the expression of various proteins that promote the production of vasodilators (13, 31, 40), increase antioxidant enzymes (9, 15, 18, 40), and decrease antagonist stimuli (17, 29, 48). Indeed, evidence exists of vascular function being augmented in old endurance trained subjects, and increased in previously sedentary old subjects by exercise training (4, 39). However, this work has been performed exclusively in the arm; therefore, although providing important insight into vascular aging, these studies may not reflect changes in NO bioavailability in the potentially more important locomotor muscles of the lower limbs, which have a predisposition to develop vascular disease (6, 30, 53).

Finally, elevated free radicals may have a deleterious effect on vascular function. Free radicals are molecules with one or two unpaired electrons that can oxidize other molecules. These oxidizing reactions can lead to decreased NO bioavailability in endothelial and vascular smooth muscle cells both by the direct destruction of NO, such as the reaction of superoxide (O_2^-) with NO to produce peroxynitrite ($OONO^-$), and by decreasing essential cofactors of NO production, such as tetrahydrobiopterin (BH_4). Oxidizing reactions are opposed by enzymatic (e.g., Superoxide dismutase) and non-enzymatic (e.g., Vitamin C) antioxidants to maintain an appropriate prooxidant/antioxidant balance. However, when the production of free radicals outstrips antioxidant defenses, the result is oxidative stress, which potentially leads to vascular dysfunction and cellular damage. Oxidative stress has been demonstrated to increase with advancing age (5, 36), and contributes to impaired vasodilation in the old (5, 51). Increased antioxidant capacity, achieved by the infusion of ascorbic acid (Vitamin C), restores endothelium-dependent vasodilation in the brachial artery of old subjects, suggesting that acute antioxidant supplementation can limit oxidative stress and improve the vasodilatory response in the old (8, 43, 44). However, currently the literature specific to the effect of antioxidant supplementation on vascular function of the lower limbs with age is lacking.

It is clear that measuring the bioavailability of NO has useful clinical implications. However, recently the validity of the most widely utilized method of non-invasively assessing NO-mediated vascular function, brachial artery flow-mediated dilation (FMD), has been called into question in terms of both its reliability and NO dependence (35, 37, 47, 52). Additionally, growing evidence that limb-specific vascular

aging may lead to greater dysfunction in the arteries of the lower limbs suggests that brachial artery FMD may not be the most appropriate measure of global vascular health (6, 30, 50). This has meant that there is a burgeoning interest in identifying alternative, clinically relevant, methods with which to assess vascular function.

PLM, an exercise model essentially devoid of increases in metabolism, and therefore metabolically-mediated vasodilation, results in a transient yet significant increase in leg blood flow (LBF) and leg vascular conductance (LVC) (12, 46). In the young, the hyperemic response to PLM has been documented to predominantly reflect NO-dependent vasodilation (11, 28, 46). Indeed, reducing NO bioavailability via nitric oxide synthase (NOS) inhibition (intra-arterial infusion of N^G-monomethyl-L-arginine, L-NMMA) greatly reduces the vasodilatory response to PLM in young males (11, 28, 46).

With healthy aging, PLM-induced vasodilation is attenuated (12, 27), and this attenuation is due mainly to a reduction in NO bioavailability (11, 45). Interestingly, the influence of NO on PLM-induced vasodilation is even more evident when utilizing alterations in body posture. In the supine posture, vascular pressures are normalized across the body, but in the upright-seated posture, gravity acts to form a hydrostatic column in the vasculature, which differentially increases arterial and venous blood pressure in the legs, such that FPP is increased. In young men, this increased FPP doubles the vasodilatory response to PLM (12), an augmentation that is almost exclusively NO mediated (11). However, due to attenuated NO bioavailability, increasing FPP in old men has no effect on the PLM-induced vasodilation (11). Taken together, these findings suggest that the vasodilatory response to PLM, with and without alterations in FPP, could be a clinically relevant tool to assess NO-mediated vascular function, as well as for

determining the mechanisms responsible for the age-associated reduction in NO bioavailability.

Accordingly, we examined PLM-induced vasodilation during alterations in FPP in young and old subjects. Specifically, the first study examined the PLM-induced vasodilatory response in healthy young and old female subjects. The second study compared the vasodilatory response to PLM in sedentary young controls and in old subjects across three levels of physical activity (sedentary, physically active, and endurance trained) to evaluate the impact that exercise has on vascular function of the lower limbs with age. The third study utilized oral antioxidant supplementation in young and old healthy sedentary subjects to assess the contribution of oxidative stress to the attenuated PLM-induced vasodilation with age. The overall objective of this dissertation was to provide insight into the mechanisms responsible for attenuated NO bioavailability and subsequently limited PLM-induced vasodilation in the lower limbs with age.

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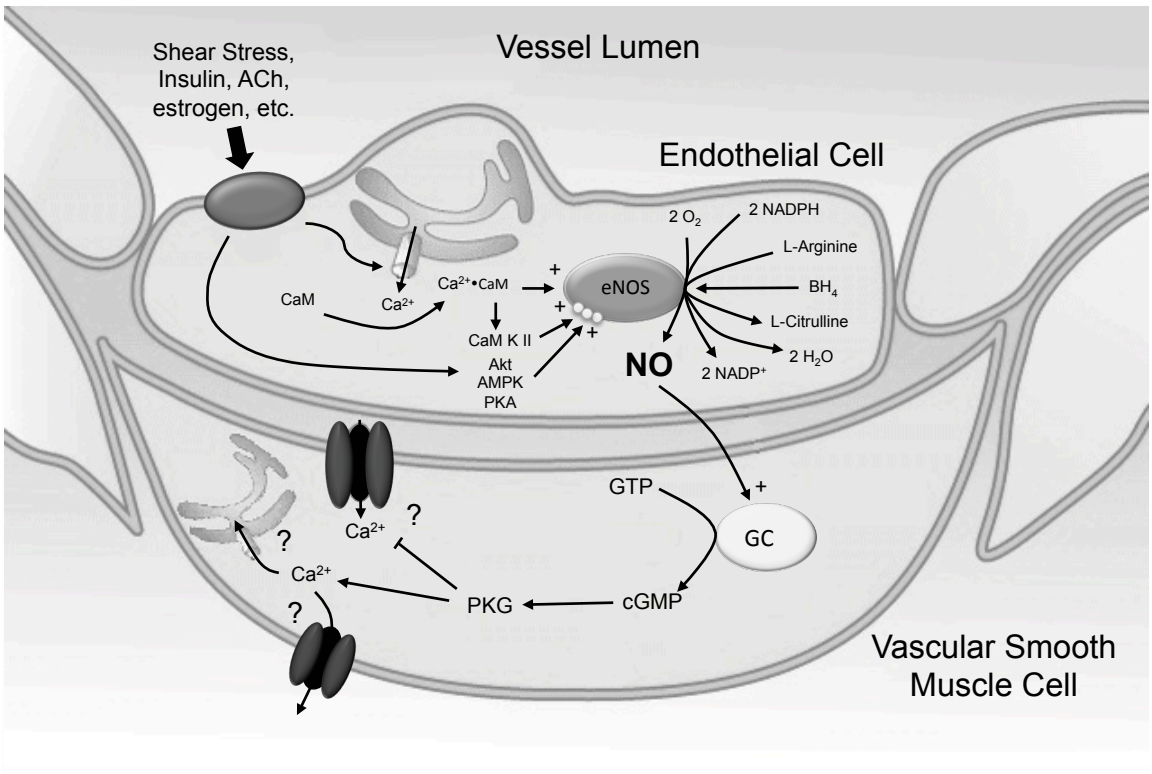

Figure 1.1 Vasodilating pathway of endothelium-derived nitric oxide. CaM, calmodulin; Ca^{2+}, calcium; CaM K II, calmodulin kinase II; AMPK, 5' adenosine monophosphate-activated protein kinase; PKA, protein kinase A; eNOS, endothelial nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; $NADP^{+}$, nicotinamide adenine dinucleotide phosphate; BH_4, tetrahydrobiopterin; NO, nitric oxide; GC, guanylyl cyclase; GTP, guanosine 5' triphosphate; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G.

CHAPTER 2

PASSIVE LEG MOVEMENT-INDUCED VASODILATION IN WOMEN: THE IMPACT OF AGE

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Passive leg movement-induced vasodilation in women: the impact of age

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Groot HJ, Rossman MJ, Trinity JD, Layec G, Ives SJ, Richardson RS. Passive leg movement-induced vasodilation in women: the impact of age. *Am J Physiol Heart Circ Physiol* 309: H995–H1002, 2015. First published July 17, 2015; doi:10.1152/ajpheart.00422.2015.—Passive leg movement (PLM), an assessment of predominantly nitric oxide-dependent vasodilation, is decreased with age and cannot be augmented by posture-induced increases in femoral perfusion pressure in older men. However, this novel method of assessing vascular function has yet to be used to evaluate alterations in nitric oxide-dependent vasodilation with age in females. PLM was performed in 10 young (20 ± 1 yr) and 10 old (73 ± 2 yr) women in both the supine and upright-seated postures, whereas central and peripheral hemodynamic measurements were acquired second by second using noninvasive techniques (finger photoplethysmography and Doppler ultrasound, respectively). The heart rate response to PLM was attenuated in the old compared with the young in both the supine (young, 10 ± 1; and old, 5 ± 1 beats/min; $P < 0.05$) and upright-seated posture (young, 10 ± 2; and old, 5 ± 1 beats/min; $P < 0.05$), leading to a blunted cardiac output response in the old in the upright-seated posture (young, 1.0 ± 0.2; and old, 0.3 ± 0.1 l/min; $P < 0.05$). The PLM-induced peak change in leg vascular conductance was lower in the old compared with the young in both postures (young supine, 5.7 ± 0.5; old supine, 2.6 ± 0.3; young upright, 9.2 ± 0.7; and old upright, 2.2 ± 0.4 ml·min^{-1}·mmHg^{-1}; $P < 0.05$) and was significantly augmented by the upright-seated posture in the young only, revealing a vasodilatory reserve capacity in the young (3.5 ± 0.6 ml·min^{-1}·mmHg^{-1}, $P < 0.05$) that was absent in the old (−0.5 ± 0.3 ml·min^{-1}·mmHg^{-1}, $P = 0.18$). These data support previous literature demonstrating attenuated PLM-induced vasodilation with age and extend these findings to include the female population, thus bolstering the utility of PLM as a novel assessment of vascular function across the life span in humans.

passive leg movement; vascular function; posture; endothelium

NEW & NOTEWORTHY

Passive leg movement (PLM) is a novel method of assessing predominantly nitric oxide mediated vascular function. PLM-induced vasodilation is attenuated with age in men; however, PLM has never before been used in the female population. Our data demonstrate that aging impairs PLM-induced vasodilation in women, thus expanding the utility of the PLM model.

RECENTLY, PASSIVE LEG MOVEMENT (PLM), a novel method for assessing vascular function, has been gaining recognition. In the supine posture, PLM initiates a vasodilatory response in young men that has been documented to be attenuated in old men (28, 39), is predominantly nitric oxide (NO) mediated, and therefore likely reflects endothelium-dependent vascular function (28, 39). Interestingly, when femoral perfusion pressure (FPP) is increased by moving from the supine to the upright-seated posture, young men exhibit an NO-mediated vasodilatory reserve capacity, whereas old men, due to diminished NO bioavailability, do not (10, 11). Likewise, the initial vasodilatory response to PLM (~9 s) differs between young and old men, with the old demonstrating a slower onset (10, 11, 38). Due to the powerful antiatherogenic properties of NO (18, 34), and the importance of intact vascular function in cardiovascular disease (CVD) prevention, an assessment such as the response to PLM, which has the potential to be used across the life span, may be of great clinical importance. However, this novel method of assessing vascular function has yet to be used to evaluate alterations in endothelium-dependent vasodilation with age in females.

Premenopausal women appear to have a reduced risk of CVD compared with both men and postmenopausal women (21). This has led to the belief that young women possess an inherent cardioprotection, which may be related to estrogen. Estrogen, which declines postmenopause, is recognized to have powerful effects on the vasculature. As a steroid hormone, estrogen is able to diffuse easily across endothelial and vascular smooth muscle cell membranes, resulting in vascular-specific genomic modifications, such as increased endothelial NO synthase (eNOS) transcription and ultimately eNOS protein expression (29) and nongenomic effects, such as eNOS activation and NADPH oxidase inhibition (29). Thus postmenopausal women would be expected to have decreased NO-mediated vascular function compared with young women, and, indeed, this has been the conclusion of studies using the brachial artery flow-mediated dilation (FMD) technique (4, 13). However, due to procedural complexities that have limited the clinical usefulness of FMD and recent questions regarding the contribution of NO to FMD (31, 45), there is a growing need to develop a clinically relevant tool that can be used to track the changes in vascular function of women across the life span.

Consequently, the purpose of this investigation was to assess vascular function in young and old women using the novel PLM test. Specifically, we hypothesized that *1*) old women would exhibit an attenuated PLM-induced vasodilation compared with young women, *2*) increasing FPP by assuming an upright-seated posture would augment the vasodilatory response in young women only, such that *3*) the vasodilatory reserve capacity would be greater in the young women than in the old women and *4*) increased FPP would augment the rapid vasodilation in the young women, with no change in the old women. If proven to be correct, such findings would support the potential clinical relevance of the PLM test for assessing vascular function in women across the life span.

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METHODS

Subjects

Twenty healthy women (10 young and 10 old) participated in this research study. Subjects were included based on a lack of overt cardiovascular or metabolic disease and aged 18–25 yr for the young and >65 yr for the old. All procedures were approved by the Institutional Review Boards of the University of Utah and the Salt Lake City Veterans Affairs Medical Center, and written informed consent was obtained from each participant before inclusion in the study. The study conformed to the standards set by the Declaration of Helsinki.

Experimental Protocol

Each subject reported to the laboratory for both a familiarization and experimental trial. Upon arrival for the familiarization trial, anthropometric measurements were performed, followed by instrumentation and PLM, both to acquaint participants with the experimental procedure and to ensure their ability to remain relaxed during a PLM protocol.

Subjects arrived on the experimental day fasted and having refrained from caffeine for 12 h prior, and exercise for 24 h before the initiation of data collection. To control for variations in circulating hormones, young females were included only if they were not taking pharmaceutical contraceptives and were then studied within the first 7 days (follicular phase) of the menstrual cycle. The old women were postmenopausal and not on hormone replacement therapy. Blood was collected from the antecubital vein to assess blood lipids, fasting glucose, C-reactive protein (CRP), ferric-reducing ability of plasma (FRAP), protein carbonyl, and hemoglobin and to perform a complete blood cell count. Subjects were then assigned to either the supine or upright-seated posture using a counterbalanced design. After instrumentation, participants rested for at least 20 min in the assigned posture before the start of data collection.

Hemodynamic measurements were collected during 1 min of baseline with the leg held at a 180° knee joint angle, followed by 2 min of passive knee flexion-extension through a 90° range of motion (180-90°) at 1 Hz. Throughout the protocol, the contralateral leg remained supported and motionless with the knee joint extended (180°). PLM was achieved by a member of the research team with real-time feedback provided by a position sensor and digital display to ensure full range of motion. A metronome, initiated before the start of baseline data collection, was used to maintain cadence. Before the start and throughout the protocol participants were encouraged to remain passive and to resist the urge to help or hinder the passive movement. To avoid the startle reflex, participants were made aware that the PLM would begin in ~1 min but were not told exactly when the movement would begin to reduce the chance of an anticipatory response (42). The protocol was repeated in the opposing body posture (supine or upright seated) after a rest period of at least 20 min in the new posture.

Baseline for all variables was determined using the average of the data for the 60 s before the initiation of PLM. Data collected during the first minute of PLM were analyzed second by second and smoothed using a 3-s rolling average before final data analysis, with 12 s-averages used for the second minute of movement.

Measurements

Central hemodynamics. Heart rate (HR) was determined using an electrocardiogram (ECG), and mean arterial pressure (MAP) was determined by finger photoplethysmography with a Finometer (Finapres Medical Systems, Amsterdam, The Netherlands) positioned at heart level. Stroke volume (SV) was calculated using the Modelflow method (Beatscope, version 1.1; Finapres Medical Systems), with cardiac output (CO) calculated as the product of SV and HR. Throughout each protocol, ECG, SV, CO, and MAP signals underwent analog-to-digital conversion and were simultaneously acquired (200 Hz) using commercially available data acquisition software (AcqKnowledge, Biopac Systems, Goleta, CA).

Peripheral hemodynamics. Measurements of blood velocity in the common femoral artery (CFA) and vessel diameter were performed in the passively moved leg distal to the inguinal ligament and proximal to the bifurcation of the superficial and deep femoral artery using a Logic 7 ultrasound system (General Electric Medical Systems, Milwaukee, WI). The Logic 7 was equipped with a linear array transducer operating at an imaging frequency of 14 MHz. CFA diameter was determined at a perpendicular angle along the central axis of the scanned area, and blood velocity was measured using the same transducer with a frequency of 5 MHz. All blood velocity measurements were obtained with the probe appropriately positioned to maintain an insonation angle of 60° or less. The sample volume was maximized according to vessel size and was centered within the vessel based on real-time ultrasound visualization. With the use of CFA diameter and mean velocity (V_{mean}; angle corrected and intensity weighted) leg blood flow (LBF) was automatically calculated by commercially available software (Logic 7) as $V_{mean}\pi(\text{vessel diameter}/2)^2 \times 60$, where blood flow is in milliliters per minute. Leg vascular conductance (LVC) was calculated as LBF divided by MAP. The peak changes in LBF and LVC (ΔLBF_{peak} and ΔLVC_{peak}, respectively) were calculated as peak minus baseline, cumulative area under the curve (LBF_{AUC} and LVC_{AUC}) was calculated as $\sum\{y_i[x_{(i+1)} - x_i] + (1/2)[y_{(i+1)} - y_i][x_{(i+1)} - x_i]\}$, and vasodilatory reserve capacity was calculated as the upright-seated ΔLVC_{peak} minus the supine ΔLVC_{peak}.

Blood assays. Glucose, lipids, and complete blood cell count were determined using standard clinical techniques. Plasma and serum samples were stored at −80° until analysis. The FRAP assay was performed to assess total antioxidant capacity, using the method described by Benzie and Strain (3), whereas oxidative stress was assessed by measuring protein carbonyl levels (Northwest Life Science Specialties, Vancouver, WA). Systemic inflammation was assessed by measuring CRP levels (R&D Systems, Minneapolis, MN). All assays were performed in duplicate.

Knee joint angle. During each protocol, knee joint angle of the passively moved leg was continuously recorded using a Vishay Spectrol 360 degree Smart Position Sensor (Vishay Intertechnology, Malvern, PA) mounted on a BREG X2K knee brace (BREG, Vista, CA) worn by the participants.

Anthropometrics. Body mass and height were recorded and used to calculate body mass index (BMI) as BMI = body mass × height^2, where body mass is measured in kilograms and height is measured in meters. Thigh volume of the passively moved leg was calculated, as previously described (19), using three measurements of thigh circumference (proximal, middle, and distal), thigh length, and skinfold measurements.

Physical activity level. Physical activity level (PAL) was assessed using both a subjective PAL recall questionnaire and objective accelerometer data. The PAL questionnaire included items regarding the average type, frequency, intensity, and duration of physical activity in any given week. After receiving standardized operating instructions, subjects wore an accelerometer (GT1M; Actigraph, Pensacola, FL) for a minimum of a continuous 7 days, with adherence automatically assessed by the device. Average total daily physical activity was expressed as both average steps per day, and average total accelerometer as counts per minute. The latter assessment was separated into sedentary, low-, moderate-, high-, and very high-intensity categories using device-specific software (Actilife, Pensacola, FL). Previous research has documented the validity and reliability of the Actigraph GT1M in terms of the estimation of daily physical activity with both steps per day and accelerometer counts per minute (1, 43). Classification of the subjects' level of physical activity, as determined by steps per day, was based on a validated

Table 1. *Subject characteristics*

	Young	Old
n	10	10
Age, yr	20 ± 1	73 ± 2#
Height, cm	162 ± 2	163 ± 1
Weight, kg	58 ± 2	69 ± 3#
Body mass index, kg/m^2	22 ± 1	26 ± 1#
Thigh volume, dl	42 ± 3	44 ± 3
CFA diameter, cm	0.71 ± 0.01	0.80 ± 0.02#
Glucose, mg/dl	71 ± 2	75 ± 2
Cholesterol, mg/dl	171 ± 12	221 ± 11#
Triglycerides, mg/dl	80 ± 9	126 ± 18
HDL, mg/dl	57 ± 3	62 ± 2
LDL, mg/dl	99 ± 10	127 ± 9
Hemoglobin, g/dl	14.1 ± 0.3	14.3 ± 0.1
White blood cell, k/μl	5.4 ± 0.4	5.4 ± 0.5
Neutrophil, k/μl	2.8 ± 0.3	3.0 ± 0.3
Lymphocyte, k/μl	2.0 ± 0.2	1.8 ± 0.1
Monocyte, k/μl	0.46 ± 0.02	0.42 ± 0.03
FRAP, mM/ml	0.90 ± 0.01	0.88 ± 0.04
Protein carbonyls, ηm/mg	0.089 ± 0.004	0.135 ± 0.010#
C-reactive protein, ηg/ml	317 ± 59	1,563 ± 249#
Activity counts/min	156 ± 21	119 ± 15
Steps/day	6,734 ± 797	5,012 ± 563

Values are means ± SE; *n*, number of subjects. CFA, common femoral artery; FRAP, ferric-reducing ability of plasmaprotein. #$P < 0.05$, significantly different from young women.

scale (sedentary, <5,000; low active, 5,000–7,499; somewhat active, 7,500–9,999; active, 10,000–12,499; and highly active, ≥12,500 steps/day) (33, 40).

Statistical Analysis

Two-way, repeated-measures ANOVA were used to determine significant differences in baseline and the absolute change from baseline to peak for HR, SV, CO, MAP, LBF and LVC, as well as AUC for LBF and LVC. Student's *t*-tests were used to compare subject characteristics. Pearson's correlations were employed to assess the strength of relationships between variables. Significance was set at an α-level of 0.05, and data are presented as means ± SE.

RESULTS

Subjects

In addition to an average age difference of over 50 yr, the old women had significantly greater body mass (~19%), BMI (~18%), total cholesterol (~30%), protein carbonyl (~52%), CRP (~393%), and larger CFA diameter (~13%) (Table 1). The groups exhibited similar total daily physical activity, as assessed by both questionnaire and accelerometry, and all subjects were categorized within the sedentary to low physical activity ranges based on the step-determined scale (Table 1) (33, 40).

Central Hemodynamics

Resting and peak changes in central hemodynamics are displayed in table 2. At rest there was no difference in SV, HR, or CO between groups in either body posture. While all subjects were normotensive (<140/90 mmHg), resting MAP was significantly higher in the old compared with the young women in both body postures ($P < 0.05$). In the young, the upright-seated posture elicited a significant decrease in SV ($P < 0.05$) and an increase in HR ($P < 0.05$) that maintained CO, whereas the same posture alterations had no effect on central hemodynamics at rest in the old.

Both groups demonstrated significant increases in SV, HR, and CO and decreases in MAP ($P < 0.05$) as a consequence of PLM. However, the ΔHR_{peak} was significantly lower in the old compared with the young in both body postures, and the ΔSV_{peak} was greater in the young with the upright-seated posture, while tending to be lower in the old with this posture ($P = 0.19$), resulting in an attenuated ΔCO_{peak} in the old women while in the upright-seated posture ($P < 0.01$). Decreases in MAP (ΔMAP_{peak}) during PLM were similar between groups and postures.

Leg Blood Flow and Leg Vascular Conductance

Resting, peak change, and AUC data for peripheral hemodynamics are displayed in Table 2. Resting LBF and LVC were

Table 2. *Central and peripheral hemodynamics*

	Supine		Upright Seated	
	Young	Old	Young	Old
		Rest		
MAP, mmHg	86 ± 2	104 ± 7#	81 ± 3	106 ± 6#
CO, l/min	4.9 ± 0.3	4.7 ± 0.5	4.9 ± 0.2	4.1 ± 0.4
SV, ml/beat	75 ± 4	80 ± 8	69 ± 2*	73 ± 8
HR, beats/min	66 ± 4	63 ± 1	71 ± 4*	64 ± 2
LBF, ml/min	351 ± 44	284 ± 29	350 ± 44	229 ± 21#*
LVC, ml·min^{-1}·mmHg^{-1}	4.0 ± 0.5	2.9 ± 0.4	4.3 ± 0.5	2.2 ± 0.2#
		Passive leg movement		
ΔMAP_{peak}, mmHg	−11 ± 1	−14 ± 2	−12 ± 1	−17 ± 2
ΔCO_{peak}, l/min	0.7 ± 0.1	1.2 ± 0.5	1.0 ± 0.2	0.3 ± 0.1#
ΔSV_{peak}, ml/beat	4 ± 1	14 ± 6	10 ± 2*	6 ± 1
ΔHR_{peak}, beats/min	10 ± 1	5 ± 1#	10 ± 2	5 ± 1#
ΔLBF_{peak}, ml/min	490 ± 34	250 ± 33#	713 ± 59*	224 ± 40#
LBF_{AUC}, ml/min	200 ± 23	79 ± 22#	284 ± 45*	67 ± 17#
ΔLVC_{peak}, ml·min^{-1}·mmHg^{-1}	5.7 ± 0.5	2.6 ± 0.3#	9.2 ± 0.7*	2.2 ± 0.4#
LVC_{AUC}, ml·min^{-1}·mmHg^{-1}	2.3 ± 0.3	0.8 ± 0.2#	3.3 ± 0.5*	0.6 ± 0.2#

Values are means ± SE; $n = 10$ old and 10 young women. MAP, mean arterial pressure; CO, cardiac output; SV, stroke volume; HR, heart rate; LBF, leg blood flow; LVC, leg vascular conductance; Δ_{peak}, peak change from baseline; AUC, area under the curve. *$P < 0.05$, significantly different from supine posture; #$P < 0.05$, significantly different from young women.

not different between groups in the supine posture. With the upright-seated posture, resting LBF was reduced in the old, such that both LBF and LVC were significantly lower in old compared with young women.

PLM in the supine and upright-seated postures resulted in a significant hyperemic response in both the young and old women ($P < 0.05$; Table 2, Figs. 1 and 2). In the supine posture, ΔLBF_{peak} and LBF_{AUC} were attenuated in the old compared with the young women (49 and 60%, respectively; Fig. 1, *B* and *C*), resulting in a similarly attenuated ΔLVC_{peak} and LVC_{AUC} (54 and 66%, respectively; Fig. 2, *B* and *C*) in the old. The upright-seated posture significantly increased the LBF ($\Delta LBF_{peak} = 46\%$, and $LBF_{AUC} = 42\%$; Fig. 1, *B* and *C*) and LVC ($\Delta LVC_{peak} = 61\%$, and $LVC_{AUC} = 43\%$; Fig. 2, *B* and *C*) response to PLM in the young, with no effect of posture in the old. This posture-induced increase in ΔLVC_{peak} in response to PLM in the young women revealed a significant vasodilatory reserve capacity (3.5 ± 0.6 ml·min^{-1}·mmHg^{-1}; $P < 0.001$) that was absent in the old (-0.5 ± 0.3 ml·min^{-1}·mmHg^{-1}; $P = 0.18$) women (Fig. 3).

In terms of total antioxidant capacity, oxidative stress, and inflammation, neither FRAP nor PC was significantly related to either ΔLVC_{peak} or LVC_{AUC}. CRP displayed a significant negative relationship to ΔLVC_{peak} in the supine and upright-seated posture ($r = 0.56$, $P < 0.01$; and $r = 0.70$, $P < 0.001$, respectively), as well as with the vasodilatory reserve capacity ($r = 0.65$, $P < 0.01$). Similarly, CRP was negatively related to both supine and upright-seated LVC_{AUC} ($r = 0.56$, $P < 0.05$; and $r = 0.65$, $P < 0.01$, respectively).

The immediate vasodilatory response (slopes of increasing LVC over time, for the first 9 s) to PLM was significantly reduced with age in both the supine (old, 0.15 ± 0.04; and young: 0.55 ± 0.04; $P < 0.001$) and the upright-seated (old, 0.16 ± 0.06; and young, 0.93 ± 0.09; $P < 0.001$) postures and was significantly elevated in the upright-seated posture, compared with supine, in the young only ($P < 0.001$; Fig. 4).

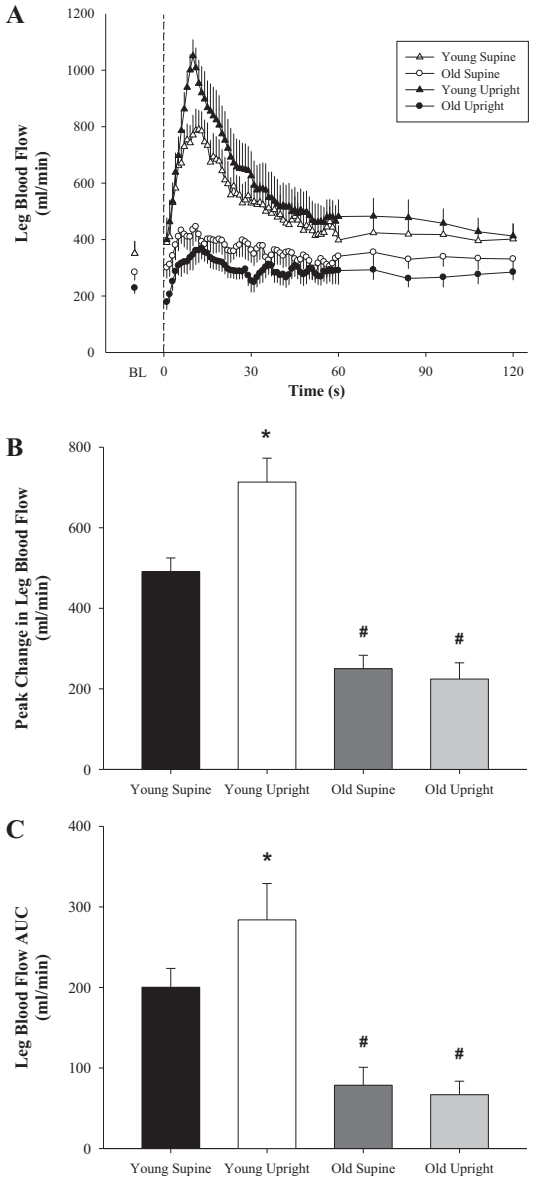

Fig. 1. Passive leg movement (PLM)-induced changes in leg blood flow (LBF) with age in women. *A*: second-by-second tracing of LBF (ml/min) at baseline (BL) and throughout 2 min of PLM. *B*: peak change in LBF from BL (ΔLBF_{peak}, ml/min); *C*: LBF area under the curve (AUC) for the first 60 s of PLM (LBF_{AUC}, ml/min). Young, $n = 10$; old, $n = 10$. BL indicates the average of the 60 s just before initiation of PLM. Dashed line at 0 s indicates the start of 2 min of PLM. *$P < 0.05$, significantly different from supine posture; #$P < 0.05$; significantly different from young women. Values are means ± SE.

DISCUSSION

The purpose of this investigation was to increase the generalizability of the PLM model by extending the previous literature to include the female population. Similar to findings in men, older women demonstrated an attenuated vasodilatory response to PLM in the supine posture compared with the young. The upright-seated posture, which significantly augmented the PLM-induced vasodilation in young women, had no effect on the vasodilatory response in the old. Similarly, in the old, the rapid vasodilatory (first 9 s) response to PLM in the supine posture was attenuated compared with the young women, and unlike in the young where the response was accelerated, the rapid vasodilatory response in the old women remained unchanged with increased FPP, thus resulting in a greater age difference in the upright-seated posture. Finally, the augmented PLM-induced ΔLVC_{peak} due to the upright-seated posture revealed a significant vasodilatory reserve capacity in young women that was absent in the old. These findings demonstrate that vascular function, assessed by the PLM model, declines with age in women. This expands the use of PLM to a wider population

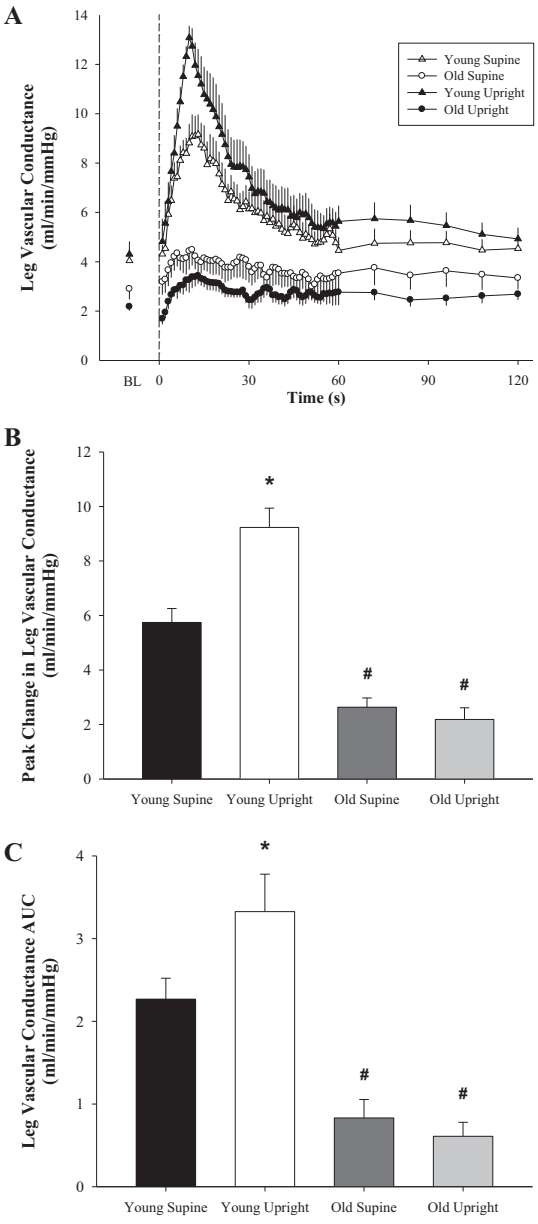

Fig. 2. PLM-induced changes in leg vascular conductance (LVC) with age in women. *A*: second-by-second tracing of LVC (ml·min^{-1}·mmHg^{-1}) at baseline and throughout 2 min of PLM. *B*: peak change in LVC from BL (ΔLVC_{peak}, ml·min^{-1}·mmHg^{-1}). *C*: LVC AUC for the first 60 s of PLM (LVC_{AUC}, ml·min^{-1}·mmHg^{-1}). Young, $n = 10$; old, $n = 10$. BL indicates the average of the 60 s just before initiation of PLM. Dashed line at 0 s indicates the start of 2 min of PLM. *$P < 0.05$, significantly different from supine posture; #$P < 0.05$, significantly different from young women. Values are means ± SE.

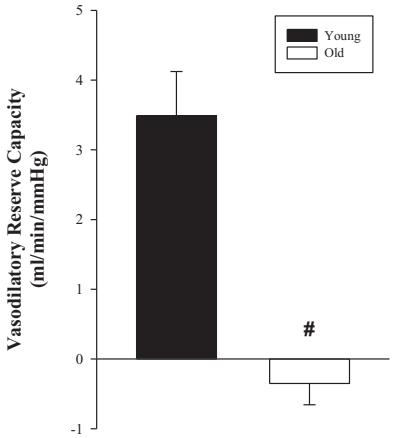

Fig. 3. PLM-induced vasodilatory reserve capacity with age in women. The difference in the PLM-induced ΔLVC_{peak} (ml·min^{-1}·mmHg^{-1}) between the supine and upright-seated posture (vasodilatory reserve capacity). Young, $n = 10$; old, $n = 10$. #$P < 0.05$, significantly different from young women. Values are means ± SE.

and bolsters the utility of PLM as a vascular function assessment across the human life span.

Age and Supine PLM-Induced Vasodilation in Women

PLM is an experimental model whereby changes in quadriceps and hamstring muscle length, without increases in metabolism, cause a significant increase in LBF and LVC. This PLM-induced hyperemia has previously been reported in both

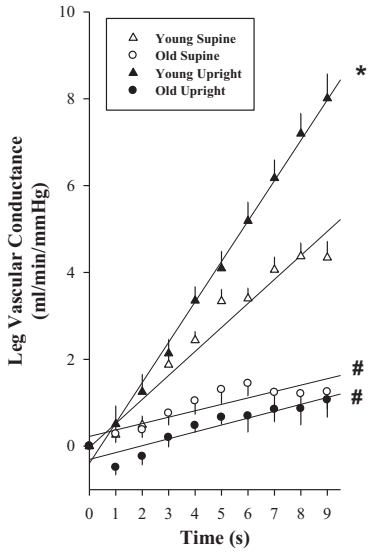

Fig. 4. PLM-induced rapid vasodilation with age in women. The rate of increase in the LVC (ml·min^{-1}·mmHg^{-1}) over time was determined for the first 9 s of PLM. Young, $n = 10$; old, $n = 10$. *$P < 0.05$, significantly different from supine posture; #$P < 0.05$, significantly different from young women. Values are means ± SE.

young and old men; however, the magnitude of the vasodilatory response to PLM is significantly attenuated with age (11, 23). Recent investigations into the mechanisms responsible for the PLM response conclude that PLM-induced vasodilation is predominantly NO mediated in young men, as evidenced by an ~80–90% reduction in the hyperemic response during intra-arterial infusion of the NO synthase (NOS) inhibitor N^G-monomethyl-L-argenine (10, 28, 39). Furthermore, by once again using NOS inhibition, both Trinity et al. (38) and Groot et al. (10) reported that the attenuated PLM-induced vasodilation described in older men was a consequence of reduced NO bioavailability with age. However, before this investigation, the PLM model had not been used to examine alterations in vascular function with age in women.

Results from the current investigation, which are strikingly similar to those previously reported in young and old men (11, 23), reveal a significant age-associated reduction in the PLM-induced ΔLBF_{peak} and ΔLVC_{peak} in the supine posture (~50%) in old women compared with young, likely because of decreased NO bioavailability (Table 2, and Figs. 1*B* and 2*B*). Of note, when individual responses were examined, the PLM-induced hyperemia was lower in all 10 of the older women compared with all, but one, of the young subjects. It should be noted that the old women had less of a HR response to PLM than the young; however, given that the PLM-induced ΔMAP_{peak} was similar between young and old, it is unlikely that this central hemodynamic difference contributed to the peripheral hemodynamic findings. This supposition is supported by the LVC data, which take MAP into account (Fig. 2). Additionally, the rapid vasodilatory response (slope of the increasing LVC over time for the first 9 s) to PLM, previously determined to be NO-independent in men in the supine posture (10, 38), was also significantly attenuated with age in women (~ 70%; Fig. 4). At the initiation of PLM, mechanical deformation of the vascular beds of the thigh may release vasodilators that act independently of the NO pathway (6, 17, 24). However, despite differences in this initial non-NO-dependent portion of the PLM response, subsequent mechanotransduction of wall shear forces likely contribute to the vasodilatory cascade making NO the dominant vasodilator for the remainder of PLM (14, 25, 30, 35).

Age and Upright-Seated PLM-Induced Vasodilation in Women

The upright-seated posture, which similarly increases FPP in young and old men (11), significantly augmented the PLM response in the young women, while having no effect on the old (Table 2, and Figs. 1 and 2). Of note, examination of the individual responses revealed that the PLM-induced hyperemia was augmented by the upright-seated posture in all ten young women, whereas the response in the old women was mixed. This posture-induced increase in vasodilation during PLM in the young enhanced the rapid vasodilation (Fig. 4) and revealed a vasodilatory reserve capacity that was absent in the old (Fig. 3).

Groot et al. (10) demonstrated that the increased rapid hyperemia with the upright-seated posture in young men could be attenuated to similar levels as the supine posture with NOS inhibition, but this rapid hyperemia was unaffected by posture in old men. This indicates that the onset of NO-mediated vasodilation during PLM is accelerated by increased FPP in the young, but not the old, and lends additional utility to the PLM model as an assessment of NO-mediated vasodilation. Similarly, whereas the upright-seated posture significantly augmented the PLM-induced rapid vasodilation in young women, the same postural alteration had no effect on the old (Table 2, and Figs. 1 and 2). In light of previous evidence that the increase in rapid vasodilation at the onset of PLM is reliant on NO, it can be inferred that this lack of an effect of the upright-seated posture on the rapid vasodilation in the old women is likely due to reduced NO bioavailability with age.

The ability for the ΔLVC_{peak} to increase when moving from the supine to upright-seated posture has been recognized to represent a vasodilatory reserve capacity (11). The upright-seated posture elicits a similar (~7 mmHg) increase in FPP in both young and old men, as measured directly in the CFA and vein (11). This augmented driving force of blood across the vascular beds of the thigh has the potential to increase endothelial shear forces at the onset of PLM, thereby resulting in a greater NO-mediated vasodilation. This was indeed the case in young men whose ΔLVC_{peak} nearly doubled in the upright-seated posture, revealing a vasodilatory reserve capacity that was nearly entirely due to NO (10). In contrast, because of reduced NO bioavailability with age, similar increases in FPP in old men did not augment the ΔLVC_{peak} in the upright-seated posture, thus a vasodilatory reserve capacity was absent in the old (10). In support of these previous findings, the young women displayed a significant vasodilatory reserve capacity, whereas the old women, who already possessed an attenuated PLM response compared with the young in the supine posture, failed to increase their ΔLVC_{peak} in the upright-seated posture and therefore lacked any vasodilatory reserve capacity (Fig. 3). Given that the vasodilatory reserve is an NO-mediated phenomena, its absence in older women provides additional evidence that NO bioavailability, as measured by PLM, is decreased with age.

Age and NO Bioavailability in Women

While it is clear that PLM-induced vasodilation is attenuated in older humans, the mechanisms leading to this age-associated decrease in NO bioavailability remain unclear. However, previous reports implicate an age-associated increase in oxidative stress and, specifically in women, the postmenopausal fall in circulating estrogen as likely causes of impaired vascular function and NO bioavailability with age.

Oxidative stress is the result of free radicals, molecules with unpaired electrons that lead to oxidizing reactions, which interfere with cellular processes resulting in damage, dysfunction, and/or apoptosis. Many of these free radicals are found in the vasculature and can interfere with NO signaling, thus diminishing NO bioavailability (5, 44). Indeed, markers of free radical production are elevated in old subjects in both endothelial cells and circulating plasma (8, 12, 41, 44) and are associated with decreased vascular function (8, 44). Interventions aimed at acutely decreasing oxidative stress, such as the infusion of the antioxidant ascorbic acid, result in improved vascular function and NO bioavailability in old subjects (9, 36, 37). In the present investigation, the older women, while not exhibiting an attenuated total antioxidant capacity, as assessed by FRAP, did have significantly elevated levels of inflamma-

tion and oxidative stress (Table 1). Additionally, although FRAP and protein carbonyls were unrelated to the PLM response, CRP was significantly related to measures of PLM-induced vasodilation ($r = 0.56–0.70$), suggesting that inflammation may play a role in the attenuated PLM response with age in women. However, further investigations into the possible effects of oxidative stress on PLM-induced vasodilation with age are necessary.

Estrogen has powerful effects on NO production, and this phenomenon is likely, at least in part, responsible for the observation that premenopausal women have decreased CVD risk compared with men and postmenopausal women. Estrogen can cause genomic modifications, such as increased transcription and ultimately the expression of eNOS protein (26, 29), As well as nongenomic effects, such as increased eNOS activation (16, 20, 26, 29). Furthermore, estrogen provides additional antioxidant effects by inhibiting NADPH oxidase and augmenting superoxide dismutase, thereby decreasing oxidative stress in the vasculature (26, 29). By these mechanisms, estrogen can decrease free radical production and NO scavenging and improve eNOS coupling and NO synthesis, resulting in greater NO bioavailability. Therefore, drastic reductions in estrogen after menopause likely contribute to decreased NO-mediated vasodilation and impaired vascular function in older women. Support for this supposition can be found by a direct comparison of the current investigation to previously published PLM results in men which suggest that women may have a greater age related impairment in vascular function (11, 23). Indeed, the magnitude of attenuation in the PLM response with age in women is 10–20% greater than that reported in men and may be potentially due to the postmenopausal fall in estrogen. Furthermore, previous investigations examining endothelium-dependent vasodilation with age in women have revealed reduced vasodilation in postmenopausal women compared with young women (4, 13) and in ovariectomized rats compared with intact animals (20, 27). These reductions in vascular function can be rescued with hormone replacement therapy (7, 15, 20, 27, 32). A direct assessment of the potential link between estrogen levels and PLM-induced hyperemia warrants further investigation.

Experimental Considerations

Because of the minimally invasive nature of this study, the direct measurement of femoral arterial, venous, and the subsequent calculation of actual perfusion pressure was not performed. Therefore, while we cannot rule out the possibility of differential FPP responses to the upright-seated posture in young and old women, a similar study performed in men reported identical posture-induced increases in FPP between the young and old subjects (11). Furthermore, while older women display some differences in orthostatic coping strategies compared with men, the responsiveness of peripheral resistance vessels appears to be similar (2), suggesting that FPP responses to the upright-seated posture are likely comparable to those previously reported in young and old men. Additionally, because infusion of a NOS inhibitor was not performed, we cannot rule out the possibility that the mechanisms for the PLM-induced vasodilation may differ between men and women. However, Mannacio et al. (22) reported that compared with young controls and age-matched men, older women displayed attenuated eNOS mRNA and a reduction in internal mammary artery relaxation in response to acetylcholine. This would suggest that attenuated NO bioavailability with age not only is an important factor in the impaired vascular function observed in older women but may play an even greater role than that observed in older men. However, future investigations into the direct role of NO in the PLM-induced vasodilation in women are needed. Additionally, as with any study focused on aging, there is often some difficulty in discerning age-related changes, such as increases in BMI and total cholesterol, from aging, per se, and that is also the case in this investigation. Finally, the relatively small sample size may have limited our ability to detect changes in certain variables (e.g., triglycerides); therefore, future studies using PLM may benefit from a larger cohort.

Conclusion

The results of the current investigation support previous findings that PLM-induced vasodilation is attenuated with age and extend these observations to include women. Additionally, increasing FPP by moving from the supine to the upright-seated posture, which magnifies the role of NO in both the rapid and overall vasodilatory response, revealed a vasodilatory reserve capacity in young, but not old women. These findings imply reduced NO bioavailability with age in women and add additional support to the utility of the PLM model as an assessment of NO-mediated vascular function across the human life span.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

H.J.G., J.D.T., and R.S.R. conception and design of research; H.J.G., M.J.R., J.D.T., G.L., and S.J.I. performed experiments; H.J.G. and M.J.R. analyzed data; H.J.G. and R.S.R. interpreted results of experiments; H.J.G. prepared figures; H.J.G. drafted manuscript; H.J.G., M.J.R., J.D.T., G.L., S.J.I., and R.S.R. edited and revised manuscript; H.J.G., M.J.R., J.D.T., G.L., S.J.I., and R.S.R. approved final version of manuscript.

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CHAPTER 3

THE EFFECT OF PHYSICAL ACTIVITY AND FITNESS ON PASSIVE LEG MOVEMENT-INDUCED VASODILATION WITH AGE

Abstract

Due to reduced nitric oxide (NO) bioavailability with age, passive leg movement (PLM)-induced vasodilation is attenuated in older sedentary subjects and, unlike the young, cannot be augmented by posture-induced elevations in femoral perfusion pressure. However, whether vascular function assessed with PLM is improved in older individuals with greater physical activity and fitness is unknown. Thus, PLM was performed on four subject groups (young sedentary (Y, 23 ± 1 yrs, n = 12); old sedentary (OS, 73 ± 2 yrs, n = 12); old active (OA, 71 ± 2 yrs, n = 10); old endurance trained (OT, 72 ± 1 yrs, n = 10)) in the supine and upright-seated posture. Hemodynamics were measured utilizing ultrasound Doppler and finger photoplethysmography. In the supine posture, PLM-induced peak change in leg vascular conductance (ΔLVC_{peak}) was significantly attenuated in the OS compared to the young (OS: 4.9 ± 0.5, Y: 6.9 ± 0.7 ml/min/mmHg), but was not different from the young in the OA and OT (OA: 5.9 ± 1.0, OT: 5.4 ± 0.4 ml/min/mmHg). The upright-seated posture significantly augmented ΔLVC_{peak} in all but the OS (OS: 4.9 ± 0.5, Y: 11.8 ± 1.3, OA: 7.3 ± 0.8, OT: 8.1 ± 0.8 ml/min/mmHg), revealing a significant vasodilatory reserve capacity in the other groups (Y: 4.92 ± 1.18, OA: 1.37 ± 0.55, OT: 2.76 ± 0.95 ml/min/mmHg). As PLM reflects predominantly NO-mediated vasodilation, these findings suggest that augmenting physical activity and fitness can protect NO bioavailability, attenuating the deleterious effects of advancing age on vascular function.

Introduction

An increased risk of cardiovascular disease (CVD) is a hallmark of advancing age. Attenuated vascular function, prior to overt signs of CVD, is an independent

predictor of future CVD risk (21, 38) and is likely a consequence of diminished endothelium-mediated vasodilation. Indeed, in sedentary older humans, endothelium-dependent vascular function is attenuated compared to young controls, whether assessed by the PLM technique (15, 16, 24, 44), intra-arterial infusion of the vasodilator acetylcholine (ACh) (2, 7, 27, 41), or brachial artery flow-mediated dilation (FMD) (4, 6, 9, 28). Specifically, it appears that the endothelium-derived vasodilator NO, which possesses antiatherogenic properties and is therefore an important factor in human aging (19, 23, 25, 32), is reduced in sedentary older subjects. However, whether this reduction in NO-dependent vascular function is the result of primary aging, or a secondary effect of reduced physical activity and fitness in the old is still unclear.

Endurance exercise-training creates an antiatherogenic arterial blood flow profile characterized by predominantly anterograde blood flow and low oscillatory shear rate (18, 20, 39, 42, 49). This beneficial flow pattern acutely increases endothelial NO synthase (eNOS) phosphorylation (12, 17), and chronically increases eNOS protein expression (17, 29), likely leading to greater NO bioavailability. However, while the current literature demonstrates a positive effect of increasing physical activity and fitness in terms of maintaining NO bioavailability with advancing age (7, 9, 27, 37), these conclusions are based exclusively on measurements made in the arm. Therefore, although providing important insight into vascular aging, these studies may not reflect changes in NO bioavailability in the potentially more important locomotor muscles of the lower limbs, which have a predisposition to develop vascular disease (8, 27, 54).

The vasodilatory response to PLM, a novel assessment of vascular function performed in the lower limb, has been documented to be predominantly (~80%) NO-

mediated in the young (26, 46), while in the old, who possess a significantly attenuated PLM-induced vasodilation (16, 24), NO seems to play little to no role (15, 44). Moreover, the vasodilatory reserve capacity (ΔLVC_{peak} from supine to upright-seated) has been demonstrated to further characterize the NO-mediated portion of the PLM response, and again reveals a clear attenuation with age (15). These findings, in addition to recent questions about the validity of brachial artery FMD as a measure of NO-bioavailability (35, 47, 54), evidence that the lower limbs of humans age in a different manner than the upper limbs (8, 27, 52), and the invasiveness of intra-arterial drug infusions, suggest that the PLM model may be a more appropriate test of vascular function. However, while the validity of the PLM model to detect reductions in NO bioavailability and vascular function with age is clear, the effect of varying activity and fitness levels in older subjects on PLM-induced vasodilation is unknown.

Therefore, the purpose of this investigation was to determine the effect of increasing levels of physical activity and fitness in older individuals on PLM-induced vasodilation in the supine and upright-seated posture in young sedentary, old sedentary, old active, and old endurance exercise-trained subjects. We hypothesized that *1)* vascular function would be attenuated in the old sedentary compared to the young sedentary group, and *2)* that increases in physical activity and fitness in the old would lead to enhanced vascular function, such that the old active group would have augmented vascular function compared to the older sedentary, and *3)* the old endurance trained would have a restoration of vascular function, demonstrating augmented vasodilation compared to both the old sedentary and old active groups, with no difference compared to the young sedentary.

Methods

Subjects

Subjects were included in this research study based on the following criteria: 1) an absence of cardiovascular or metabolic disease, as assessed by a health history questionnaire; 2) aged 18-25 yrs for the young, and greater than 65 yrs for the old; and 3) sedentary/low physical activity for the young, and three levels of physical activity in the old (sedentary/low activity, active, endurance trained). Forty-four healthy men met the inclusion criteria for this research study (young sedentary n = 12, old sedentary n = 12, old active n = 10, and old endurance trained n = 10). All procedures were approved by the Institutional Review Boards of the University of Utah and the Salt Lake City VA Medical Center, and written informed consent was obtained from each participant prior to inclusion in the study. The study conformed to the standards set by the Declaration of Helsinki.

Experimental Protocol

Subjects reported to the laboratory on two separate experimental days. On the first experimental day, subjects arrived at the laboratory after a light meal. They then performed a graded exercise test on a cycle ergometer to volitional exhaustion, during which expiratory gasses were collected to determine peak volume of oxygen consumption (VO_{2peak}). Prior to departure from the laboratory, the subjects were given an accelerometer and instructed on proper operating procedures. All subjects wore the accelerometers for a minimum of 7 consecutive days, and returned them on the second experimental day.

The second experimental day took place 7 days after the initial trial. Subjects

arrived on the second experimental day fasted and having refrained from caffeine and exercise for 24 hours prior to the initiation of data collection. Accelerometers were turned in and blood was collected from the antecubital vein to assess blood lipids, fasting glucose, and hemoglobin.

Subjects were then assigned to begin assessments in either the supine or upright-seated posture using a counterbalanced design. Subjects rested for a minimum of 20 minutes after instrumentation. Hemodynamic measurements were then collected during 1 min of baseline with the leg held at a constant 180° knee joint angle, followed by 1 min of passive knee flexion-extension through a 90° range of motion (180-90°) at 1 Hz. Throughout the protocol, the contralateral leg remained supported and motionless with the knee joint extended. PLM was achieved by a member of the research team, with real-time feedback provided by a position sensor and digital display to ensure full range of motion. A metronome, initiated before the start of baseline data collection, was used to maintain cadence. Prior to the start and throughout the protocol, participants were encouraged to remain passive and to resist the urge to help or hinder the passive movement. To avoid the startle reflex, participants were made aware that the passive movement would begin in approximately 1 min, but were not told exactly when the movement would begin in order to reduce the chance of an anticipatory response (50). The protocol was repeated in the opposing body posture (supine or upright-seated) after a rest period of at least 20 min in the new posture.

Measurements

Central hemodynamics. Heart rate (HR) was determined using an electrocardiogram (ECG), and mean arterial pressure (MAP) was determined by finger

photoplethysmography with a Finometer (Finapres Medical Systems, Amsterdam, The Netherlands) positioned at heart level. Stroke volume (SV) was automatically calculated using the Modelflow method (Beatscope, version 1.1; Finapres Medical Systems), with cardiac output (CO) calculated as the product of SV and HR.

Peripheral hemodynamics. Measurements of blood velocity in the common femoral artery (CFA) and vessel diameter were performed in the passively moved leg distal to the inguinal ligament and proximal to the bifurcation of the superficial and deep femoral artery using a Logic 7 ultrasound system (General Electric Medical Systems, Milwaukee, WI, USA). The Logic 7 was equipped with a linear array transducer operating at an imaging frequency of 14 MHz. CFA diameter was determined at a perpendicular angle along the central axis of the scanned area, and blood velocity was measured using the same transducer with a frequency of 5 MHz. All blood velocity measurements were obtained with the probe appropriately positioned to maintain an insonation angle of 60 degrees or less. The sample volume was maximized according to vessel size and was centered within the vessel based on real-time ultrasound visualization. Using CFA diameter and mean velocity (V_{mean}) (angle corrected, and intensity weighted), leg blood flow (LBF) was automatically calculated by commercially available software (Logic 7) as $V_{mean}\pi(\text{vessel diameter}/2)^2 \times 60$, where blood flow is in milliliters per minute. The PLM-induced peak change in leg blood flow (ΔLBF_{peak}) was calculated as peak LBF minus baseline LBF. Leg vascular conductance (LVC) was calculated as LBF divided by MAP. Rapid vasodilation was interpreted as the slope of increasing LVC over time for the first 9 s of PLM. Vasodilatory reserve capacity was calculated as upright-seated ΔLVC_{peak} minus supine ΔLVC_{peak}. The rapid vasodilatory

reserve capacity was calculated as the upright-seated LVC slope minus the supine LVC slope. Both the vasodilatory reserve capacity and the rapid vasodilatory reserve capacity have previously been reported to reflect NO-mediated vasodilatory mechanisms (15).

Knee joint angle. During each PLM protocol, knee joint angle of the passively moved leg was continuously recorded using a Vishay Spectrol 360 degree Smart Position Sensor (Vishay Intertechnology Inc., Malvern, PA, USA) mounted on a BREG X2K knee brace (BREG Inc., Vista, CA, USA) worn by the participants.

Anthropometrics. Body mass and height were recorded and used to calculate body mass index (BMI) as BMI = body mass x height^2, where body mass is measured in kilograms and height is measured in meters. Thigh volume of the passively moved leg was calculated, as previously described (22), using three measurements of thigh circumference (proximal, middle, and distal), thigh length, and skinfold measurements.

Physical activity and fitness levels. Physical activity level (PAL) was assessed using both a modified Minnesota PAL recall questionnaire (31) and objective accelerometer data. The PAL questionnaire included items regarding the average type, frequency, intensity, and duration of physical activity in any given week. After receiving standardized operating instructions, subjects wore an accelerometer (GT1M; Actigraph, Pensacola, FL, USA) for a minimum of 7 continuous days, with adherence automatically assessed by the device. Average total daily physical activity was expressed as both average steps per day, and average total accelerometer counts per minute. The later assessment was separated into sedentary, low, moderate, high, and very high intensity categories using device specific software (Actilife, Pensacola, FL, USA). Previous research has documented the validity and reliability of the Actigraph GT1M in the

estimation of daily physical activity (1, 51). Older subjects were initially separated into the three physical activity groups (sedentary/low activity, active, endurance trained) based on their responses to the PAL questionnaire. Grouping was then confirmed utilizing both steps per day and accelerometer counts per minute (36, 48).

Fitness level was determined by a cycling VO_{2peak} test performed on an electronically braked cycle ergometer (Lode, Groningen, The Netherlands), and pulmonary VO_2 was measured continuously throughout the test (Parvomedics, Sandy, UT). The VO_{2peak} protocol consisted of a 1 min warm-up at 25 watts followed by an incremental increase of 25 watts each minute until volitional exhaustion (3). Criteria for successful attainment of VO_{2peak} were a respiratory exchange ratio of >1.1, and the achievement of a maximal HR within 10 beats/min of the predicted value (220-age). VO_{2peak} was considered the highest 30 s average prior to cessation of exercise. Results from the cycling VO_{2peak} test provided additional confirmation of appropriate subject grouping.

Data Acquisition and Statistical Analysis

Throughout each protocol ECG, SV, CO, MAP, and knee joint angle signals underwent analog-to-digital conversion and were simultaneously acquired (200 Hz) using commercially available data acquisition software (AcqKnowledge, Biopac Systems Inc., Goleta, CA, USA). CFA diameter and V_{mean} were acquired on the ultrasound system (GE Logic 7). Baseline was analyzed using the average of the 60 s prior to the initiation of PLM. All variables were analyzed second-by-second for the 60 s of passive movement, and data were smoothed using a 3 s rolling average prior to final data analysis. Multiple 2x4 repeated measures ANOVA were used to determine significant differences in

baseline and the absolute change from baseline to peak for HR, SV, CO, MAP, LBF, LVC, and LVC slope, and Tukey's HSD was used for post hoc analysis. A one-way ANOVA (1x4) was used to determine group differences in vasodilatory and rapid vasodilatory reserve capacity. Student's t-tests were used for subject characteristics and significance was set at an α-level of 0.05. Data are presented as mean ± SEM.

Results

Subjects

The differences in the subject characteristics between groups, displayed in Table 3.1, were anticipated based on their respective physical activity and fitness levels.

Physical activity and fitness comparisons are displayed in Table 3.2 and Figure 3.1. Total daily physical activity, as assessed by both accelerometer counts/min and steps/day, was not different between young and old sedentary subjects (Figure 3.1, A and B). As previously reported (16), the similar total daily physical activity between these two groups was achieved in differing ways. Old sedentary subjects spent more time engaged in low intensity physical activity (~ 60 min/day), while young subjects spent more time in the moderate intensity category (~ 20 min/day, Table 3.2). In contrast, both absolute and relative VO_{2peak} were attenuated in the old compared to the young sedentary subjects (Figure 3.1, C and D). The old active subjects had greater activity counts/min and steps/day compared to both the young and old sedentary, greater time spent in the moderate and vigorous intensity categories compared to old sedentary (Table 3.2), and a higher VO_{2peak} than the old sedentary, but still demonstrated attenuated absolute and relative VO_{2peak} compared to the young sedentary subjects (Figure 3.1, C and D). The old endurance trained group had significantly greater activity counts/min, steps/day, time

spent in the moderate and vigorous intensity categories, and absolute and relative VO_{2peak} compared to both the young and old sedentary subjects. While the old active and old endurance trained groups took a similar number of steps/day, the old endurance trained group tended ($p = 0.06$) to spend more time in the vigorous intensity category compared to the old active, resulting in greater activity counts/min, and an augmented absolute and relative VO_{2peak} compared to the old active (Figure 3.1, B, C and D).

Central and Peripheral Hemodynamics During Rest and PLM

Central hemodynamics. At rest, MAP, CO, SV, and HR were similar between groups and body postures (Table 3.3). In response to PLM, the old trained group had less of an increase in HR in both the supine and upright-seated postures compared to the old sedentary. All other central hemodynamic responses to PLM were similar between groups and body postures.

Peripheral hemodynamics. At rest, the old sedentary group had lower LBF and LVC compared to the young in both the supine and upright-seated postures (Table 3.3). In response to PLM, the ΔLBF_{peak} in the supine posture was not different between these groups, although tending to be lower in the old sedentary compared to the young subjects ($p = 0.11$, Table 3.3). Similarly, the ΔLVC_{peak} was not different between the three old groups, or when comparing the young to the old active and old endurance trained groups, but was significantly lower in the old sedentary compared to the young (Table 3.3, Figure 3.2C). The upright-seated posture augmented the PLM response (ΔLBF_{peak} and ΔLVC_{peak}) in the young, old active, and old endurance trained groups, with no effect in the old sedentary ($p = 0.93$ and $p = 0.73$, respectively), such that ΔLBF_{peak} and ΔLVC_{peak} were significantly greater in the young, old active, and old endurance trained compared to

the old sedentary (Table 3.3, Figure 2D). However, while the old active and old endurance trained groups displayed an upright-seated posture-induced increase in the PLM response, the ΔLBF_{peak} and ΔLVC_{peak} remained attenuated compared to the young sedentary subjects.

The young, old active, and old endurance trained groups all demonstrated a significant vasodilatory reserve capacity (young sedentary: 4.92 ± 1.18, old active: 1.37 ± 0.55, old endurance trained: 2.76 ± 0.95 ml/min/mmHg; $p < 0.05$), while the old sedentary group was unable to take advantage of the posture-induced increase in FPP in terms of vasodilation (old sedentary: -0.34 ± 0.41; $p = 0.73$; Figure 3.3). Compared to the young, the old sedentary and old active groups displayed an attenuated vasodilatory reserve capacity, while the old endurance trained group's response was not significantly different from the young ($p = 0.27$). Due to the complete lack of a vasodilatory reserve capacity in the old sedentary group, both the old active and old endurance trained groups had a significantly greater vasodilatory reserve capacity compared to the old sedentary.

The rapid vasodilatory response in the supine posture was attenuated in the old sedentary compared to the young (young sedentary: 0.69 ± 0.08, old sedentary: 0.38 ± 0.06 ml/min/mmHg/s; $p < 0.05$; Figure 3.4A), with no other differences between groups (old active: 0.55 ± 0.10, old endurance trained: 0.50 ± 0.06 ml/min/mmHg/s; $p > 0.05$). Moving from the supine to the upright-seated posture significantly augmented the rapid vasodilation in the young, old active, and old endurance trained (1.08 ± 0.14, 0.75 ± 0.10, 0.86 ± 0.13 ml/min/mmHg/s, respectively; $p < 0.05$ for all), with no change in the old sedentary group (0.42 ± 0.06 ml/min/mmHg/s; $p = 0.44$), such that the rapid vasodilation in the upright-seated posture in the old sedentary was attenuated compared to all three

groups (Figure 3.4B). Despite the posture-induced increase in rapid vasodilation in the old active, the response in the young was of a greater magnitude, leading to a significant difference between these two groups. However, there was no difference in the rapid vasodilation between the young sedentary and old endurance trained subjects while in the upright-seated posture (p = 0.17). When comparing the posture-induced increase in the LVC slope between groups (rapid vasodilatory reserve capacity, Figure 3.4C), a similar qualitative pattern was observed as that for the vasodilatory reserve capacity. While there were no significant differences between the groups, both the young and old endurance trained tended to display a greater rapid vasodilatory reserve capacity compared to the old sedentary (p = 0.10 and p = 0.11, respectively).

Discussion

Utilizing the novel PLM assessment, we sought to elucidate the impact of increased physical activity and fitness on vascular function with advancing age. Among the old subjects, elevated levels of physical activity and fitness had a positive effect on PLM-induced vasodilation in the upright-seated posture. Furthermore, the vasodilatory reserve capacity and rapid vasodilatory reserve capacity appear to be affected in a dose-dependent manner by physical activity and fitness, resulting in significantly improved vascular function in the old active and old endurance trained, compared to their sedentary peers. Finally, while the upright-seated ΔLVC_{peak} in the old endurance trained was not completely restored to the level of the young, both the vasodilatory reserve capacity and rapid vasodilatory reserve capacitiy, reported to be highly NO-dependent, were similar between these two groups. Consequently, utilizing the novel PLM assessment of vascular function, these findings suggest that increasing levels of physical activity and fitness can

improve NO bioavailability and reduce the deleterious effects of advancing age on vascular function.

Age and Attenuated NO-Mediated Vascular Function

NO is an endothelium derived vasodilator that is synthesized in response to a number of stimuli, including endothelial cell deformation and luminal shear forces (10, 11, 40). In addition to its role as a vasodilator, NO prevents atherogenesis by inhibiting platelet aggregation, white blood cell adhesion, and vascular smooth muscle cell migration and proliferation (23, 25). Therefore, NO is a critically important molecule in limiting the progression of CVD, suggesting that measuring NO bioavailability may provide insight into CVD risk across the human lifespan.

A preponderance of evidence collected in the human arm demonstrates that healthy aging is associated with reduced NO-mediated vascular function (2, 7, 26, 29, 41, 44). Indeed, when the endothelium- and NO-dependent vasodilator ACh is infused into the brachial artery of young and old sedentary subjects, the vasodilatory response is attenuated in the old (7, 41). In contrast, infusion of sodium nitroprusside, an endothelium- and NO-independent vasodilator, results in similar levels of vasodilation in both young and old subjects, indicating that the attenuated vasodilation with age is a consequence of diminished endothelial NO bioavailability (7, 41). Seemingly in agreement with these findings, the percent dilation of the brachial artery in response to post-occlusion-induced increases in wall shear stress (FMD), demonstrate that the vasodilatory response is attenuated with age (6, 9, 28). However, brachial artery remodeling with age leads to increased lumen diameter in old subjects (4, 14), which confounds the calculation of FMD as a percentage change from baseline and reduces

endothelial shear forces that cause the brachial artery to dilate. Indeed, when brachial artery dilation is normalized for the shear stimulus, the difference between young and old frequently disappears (28, 53), which would suggest that vascular function is preserved with age, a finding that contradicts data from investigations using ACh infusions. Furthermore, the current literature questions the role of brachial artery FMD as a marker of NO-mediated vasodilation (35, 47, 54), and indicates that the lower limbs, which are more susceptible to age-related vascular dysfunction, may be a more appropriate site to study alterations in NO bioavailability with age (8, 27, 52).

PLM, a leg movement model essentially devoid of increases in metabolism and therefore metabolically-mediated vasodilation causes a robust vasodilation-induced increase in LBF and LVC, the magnitude of which is attenuated with age (16, 24). When the eNOS inhibitor N^G-monomethyl-$_L$-arginine ($_L$-NMMA) is infused into the common femoral artery during PLM, the vasodilatory response is reduced by ~80% in the young, indicating that the PLM-induced vasodilation is predominantly NO-mediated (15, 26, 46). Furthermore, eNOS inhibition has little effect on the already attenuated PLM response in old sedentary subjects, suggesting that reduced NO bioavailability is largely responsible for the age-related decrease in vascular function assessed by this novel approach (15, 46). The current investigation once again supports this previous work by demonstrating an age-associated attenuation in the PLM-induced ΔLBF_{peak} and ΔLVC_{peak} in the old sedentary men in both the supine and upright-seated postures (Table 3.3, Figure 3.2). Furthermore, the vasodilatory reserve capacity and the rapid vasodilatory reserve capacity, both of which are suggested to be primarily NO-dependent (15), were essentially absent in the old sedentary men, providing additional evidence in support of

the suggestion that the attenuated PLM response with age is due to reduced NO bioavailability. However, whether the PLM-induced vasodilation is protected in older individuals who maintain a greater level of physical activity and fitness has not previously been investigated.

Impact of Physical Activity and Fitness on Vascular Function with Age

Aging is associated with increased formation of atherosclerotic plaques, which may result, in part, from alterations in the arterial blood flow profile towards a proatherogenic state (5, 30, 45, 55). This proatherogenic blood flow profile is characterized by lower anterograde flow, higher retrograde flow, and greater endothelial oscillatory shear forces, all of which lead to attenuated NO bioavailability and impaired endothelium-dependent vasodilation (20, 39, 43, 49). An acute bout of endurance exercise increases anterograde blood flow, decreases retrograde blood flow, and decreases oscillatory shear both during and directly after the exercise bout (18, 42). Therefore, endurance exercise creates an antiatherogenic blood flow pattern that has been documented to acutely increase eNOS phosphorylation and chronically increase eNOS protein expression, leading to greater NO bioavailability (10, 14, 17, 29). Indeed, the forearm vascular conductance response to doubling doses of ACh is very similar between old endurance trained and young sedentary men (7, 27), and 3 months of exercise training in previously sedentary old men can restore their ACh response to that of young sedentary and old endurance trained men (7). However, results in terms of the effect of endurance exercise training in the old on brachial artery FMD are mixed, with some reporting improved vasodilation (9, 33), and others reporting no change (4, 34). These contradictory findings may be due to the confounding effects of arterial remodeling in

response to chronic exercise training that results in a greater baseline luminal diameter (13, 14). Indeed, in the current investigation, the old endurance trained tended to have a larger CFA diameter compared to their sedentary peers ($p = 0.07$, Table 3.1), but as PLM does not rely upon changes in CFA diameter, per se, to assess vascular function, this is not a confounding issue in the current study.

Utilizing the PLM model, the impact of physical activity and fitness on vascular function is clear. Both the active and endurance trained old groups exhibited significantly augmented PLM-induced ΔLVC_{peak}, vasodilatory reserve, and upright-seated rapid vasodilation compared to the old sedentary (Figures 3.2, 3.3, and 3.4, respectively). Furthermore, to our knowledge, this is the first study to assess two different levels of physically active old subjects, allowing the effect of a physical activity and fitness "dose response" on vascular function to be assessed. In this regard, in terms of the PLM-induced ΔLVC_{peak}, it would appear that higher levels of physical activity and fitness in the old endurance trained group had no additional benefit on vascular function above and beyond that exhibited by the old active group (Figure 3.2). However, the vasodilatory reserve capacity, which remained attenuated in the old active compared to the young, was improved in the old endurance trained to the point that this group's vasodilatory reserve capacity was no longer different from the young, suggesting that there is some additional augmentation of NO-mediated vasodilation with greater physical activity and fitness in the old (Figure 3.3).

Further evidence from the assessment of the rapid vasodilatory response supports the restorative or protective role of greater levels of physical activity and fitness in the old. Specifically, while both the old active and old endurance trained displayed a posture-

induced augmentation of the rapid vasodilatory response, this response was only similar in the young and old endurance trained subjects (Figure 3.4B). Furthermore, analysis of the rapid vasodilatory reserve capacity provides an additional examination of the effect of posture on the rapid vasodilatory response (Figure 3.4C). While this method of analysis created additional variance, resulting in no statistically significant differences between groups, it appears that there is a stepwise increase in the rapid vasodilatory reserve capacity with greater levels of physical activity and fitness among the old groups, such that the response in old endurance trained tended to be greater than the old sedentary, and was very similar to the young.

Together, these findings indicate that the reduction in NO bioavailability with age can be mitigated in older populations who possess greater levels physical activity and fitness. Additionally, the PLM assessment of vascular function, which is not confounded by age and chronic exercise-induced changes in arterial structure, is capable of detecting alterations in vascular function among old individuals of varying physical activity and fitness levels.

Conclusion

Utilizing the novel PLM assessment of vascular function, the results of the current investigation support previous findings that aging has a deleterious effect on vascular function in otherwise healthy, sedentary humans. Furthermore, this study adds to the literature by demonstrating that higher levels of physical activity and fitness can partially restore or protect against this age-associated decrease in PLM-induced vasodilation. The observation that the upright-seated ΔLVC_{peak}, PLM-induced vasodilatory reserve capacity, and rapid vasodilatory reserve capacity are predominantly NO dependent in the

young suggests that the reduced vascular function with age is due to decreased NO bioavailability, which can be protected by increasing levels of physical activity and fitness with advancing age.

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Table 3.1 Subject characteristics

	Young	Old Sedentary	Old Active	Old Trained
n	12	12	10	10
Age (y)	23 ± 1	73 ± 2#	71 ± 2#	72 ± 1#
Height (cm)	176 ± 2	176 ± 1	180 ± 2	177 ± 1
Weight (kg)	75 ± 3	82 ± 4	77 ± 3	71 ± 1§
BMI (kg/m^2)	24.2 ± 0.9	26.4 ± 1.1	24.0 ± 0.6	22.6 ± 0.4§
Thigh Volume (dl)	70 ± 3	65 ± 3	66 ± 3	67 ± 2
CFA Diameter (cm)	0.90 ± 0.02	1.01 ± 0.05#	1.10 ± 0.06#	1.15 ± 0.04#
Glucose (mg/dl)	72 ± 3	73 ± 3	81 ± 3	90 ± 4#§
Cholesterol (mg/dl)	163 ± 12	182 ± 8	178 ± 8	197 ± 11
Triglycerides (mg/dl)	114 ± 29	93 ± 11	73 ± 6	56 ± 2§†
HDL (mg/dl)	45 ± 3	50 ± 3	54 ± 4#	86 ± 5#§†
LDL (mg/dl)	101 ± 9	117 ± 7	113 ± 7	111 ± 6
Haemoglobin (g/dl)	15.8 ± 0.3	15.3 ± 0.2	15.0 ± 0.4	14.7 ± 0.3#
Maximum Work Rate (W)	235 ± 11	175 ± 14#	230 ± 16§	275 ± 19§

BMI, body mass index; CFA diameter, common femoral artery diameter; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol. # $p < 0.05$, significantly different from the young; § $p < 0.05$, significantly different from the old sedentary; † $p < 0.05$, significantly different from the old active. Values are mean ± SEM.

Table 3.2 Intensity of physical activity

	Young	Old Sedentary	Old Active	Old Trained
Sedentary (min/day)	1217 ± 20	1178 ± 25	1189 ± 16	1206 ± 11
Light (min/day)	186 ± 19	249 ± 24#	195 ± 18	156 ± 9§
Moderate (min/day)	34 ± 3	12 ± 3#	45 ± 6§	56 ± 8#§
Vigorous (min/day)	2 ± 1	1 ± 0	9 ± 4§	21 ± 4#§
Very Vigorous (min/day)	0.7 ± 0.5	0.5 ± 0.5	1.1 ± 1.0	0.9 ± 0.3

Average time spent in five physical activity categories per day (min/day). Young sedentary n = 12, old sedentary n = 12, old active n = 10, old endurance trained n = 10. # $p < 0.05$, significantly different from the young; § $p < 0.05$, significantly different from the old sedentary. Values are mean ± SEM.

Table 3.3 Central and peripheral hemodynamics during rest and passive leg movement (PLM) in the supine and upright-seated posture with age and across physical activity and fitness levels

Rest

	Young		Old Sedentary		Old Active		Old Trained	
	Supine	**Upright**	**Supine**	**Upright**	**Supine**	**Upright**	**Supine**	**Upright**
MAP (mmHg)	86 ± 1	86 ± 2	92 ± 3	92 ± 4	97 ± 6	96 ± 3	94 ± 3	97 ± 3
CO (l/min)	5.6 ± 0.3	5.6 ± 0.2	5.1 ± 0.5	4.9 ± 0.4	5.0 ± 0.8	4.3 ± 0.4	4.0 ± 0.4	4.2 ± 0.3
SV (ml/beat)	99 ± 5	95 ± 5	94 ± 10	91 ± 9	98 ± 18	84 ± 8	83 ± 11	86 ± 10
HR (beat/min)	57 ± 3	59 ± 2	56 ± 3	58 ± 3	51 ± 3	53 ± 3	47 ± 3	50 ± 3
LBF (ml/min)	367 ± 33	359 ± 47	230 ± 19#	245 ± 21#	339 ± 53	335 ± 43	320 ± 45	267 ± 29
LVC (ml/min/mmHg)	4.3 ± 0.4	4.3 ± 0.7	2.6 ± 0.2#	2.7 ± 0.3#	3.4 ± 0.4	3.5 ± 0.4	3.4 ± 0.5	2.9 ± 0.3

Passive Leg Movement (Δpeak)

	Young		Old Sedentary		Old Active		Old Trained	
	Supine	**Upright**	**Supine**	**Upright**	**Supine**	**Upright**	**Supine**	**Upright**
ΔMAPpeak (mmHg)	-6 ± 2	-5 ± 1	-6 ± 2	-6 ± 2	-11 ± 2	-7 ± 1	-6 ± 1	-7 ± 1
ΔCOpeak (l/min)	1.4 ± 0.2	1.1 ± 0.2	1.3 ± 0.3	1.2 ± 0.3	0.8 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.2
ΔSVpeak (ml/beat)	13 ± 2	11 ± 1	9 ± 2	12 ± 2	8 ± 3	7 ± 2	8 ± 3	6 ± 2
ΔHRpeak (beat/min)	11 ± 2	7 ± 2	16 ± 5	12 ± 5	7 ± 1	8 ± 3	5 ± 2§	3 ± 1§
ΔLBFpeak (ml/min)	577 ± 58	950 ± 87*	446 ± 49	431 ± 51#	489 ± 56	678 ± 72#§*	481 ± 43	728 ± 61#§*
ΔLVCpeak (ml/min/mmHg)	6.9 ± 0.7	11.8 ± 1.3*	4.9 ± 0.5#	4.6 ± 0.5#	5.9 ± 1.0	7.3 ± 0.8#§*	5.4 ± 0.4	8.1 ± 0.8#§*

MAP, mean arterial pressure; CO, cardiac output; SV, stroke volume; HR, heart rate; LBF, leg blood flow; LVC, leg vascular conductance; Δ_{peak}, peak change from baseline. Young sedentary n = 12, old sedentary n = 12, old active n = 10, old endurance trained n = 10. * $p < 0.05$, significantly different from the supine posture. # $p < 0.05$, significantly different from the young; § $p < 0.05$, significantly different from the old sedentary. Values are mean ± SEM.

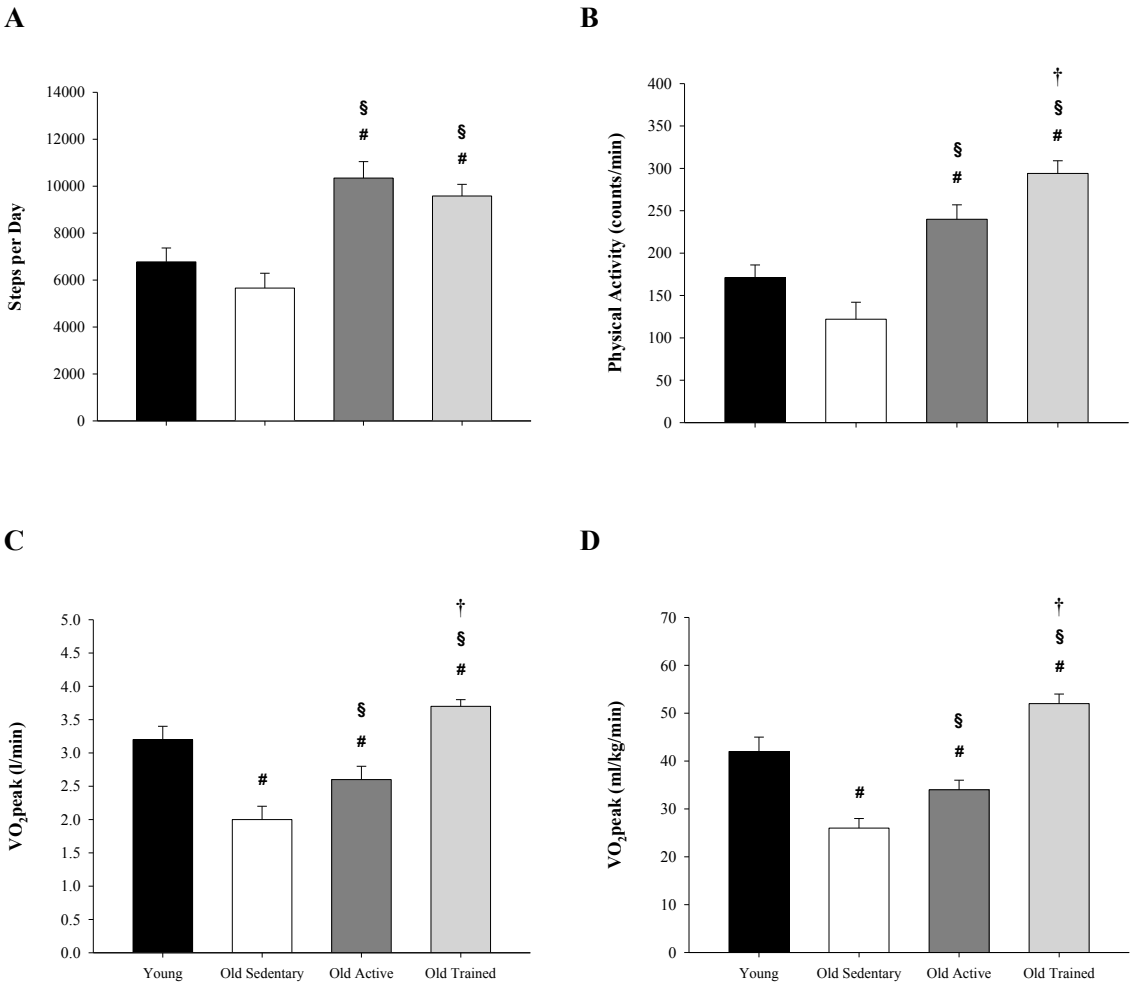

Figure 3.1 Physical activity and fitness levels. Average steps taken per day (*A*); Total daily physical activity (*B*, activity counts/min); both steps per day and total daily physical activity were assessed as the average from at least 7 days of accelerometry; Absolute (*C*, l/min) and relative (*D*, ml/kg/min) volume of oxygen consumed at peak exercise during a graded cycling test to volitional exhaustion. Young sedentary n = 12, old sedentary n = 12, old active n = 10, old endurance trained n = 10. # $p < 0.05$, significantly different from the young; § $p < 0.05$, significantly different from the old sedentary; † $p < 0.05$, significantly different from the old active. Values are mean ± SEM.

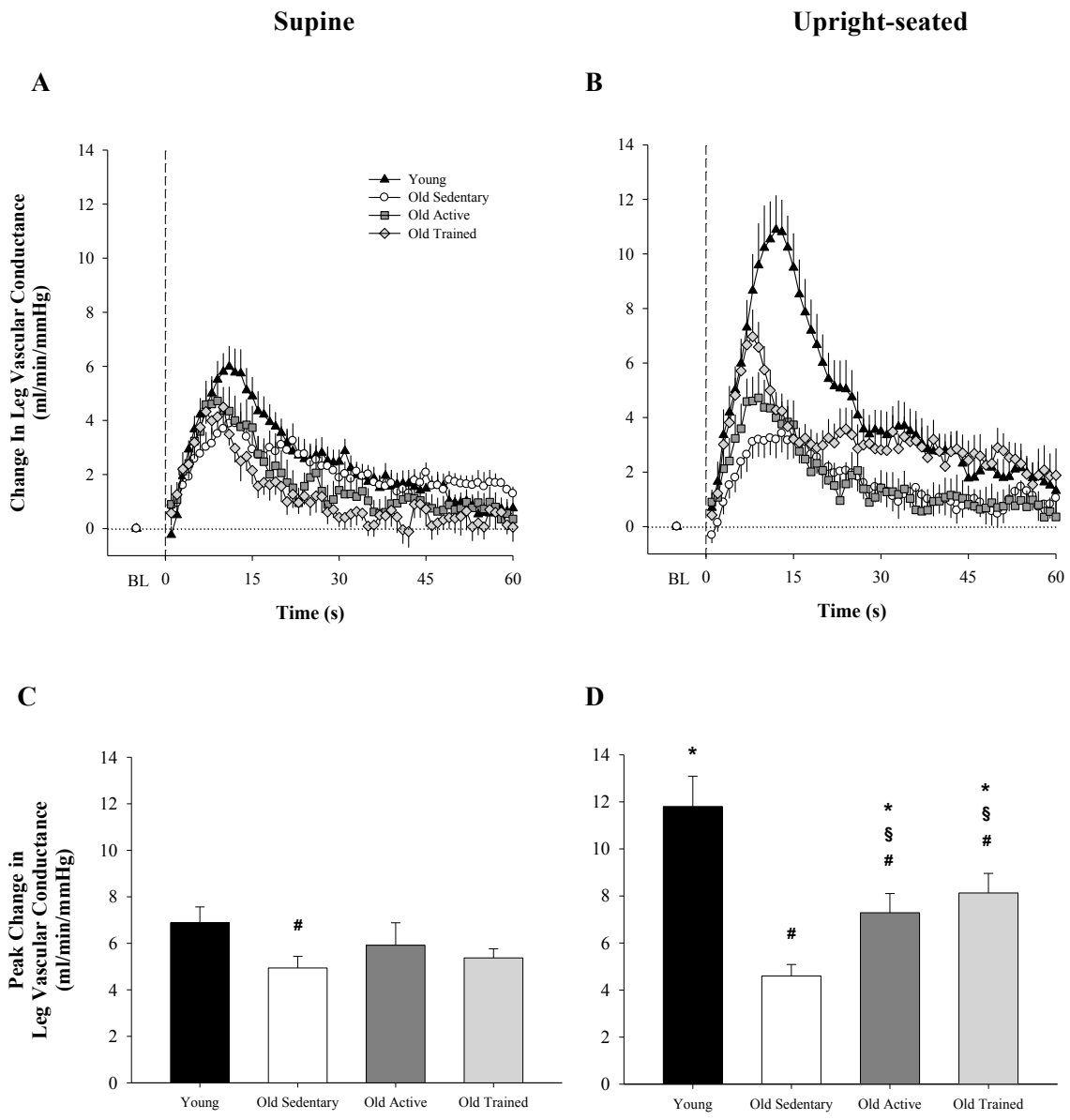

Figure 3.2 Passive leg movement (PLM)-induced changes in leg vascular conductance in the supine and upright-seated posture with age and across physical activity and fitness levels. Second-by-second tracing of the change in leg vascular conductance (ml/min/mmHg) throughout 1 min of PLM in the supine (*A*) and upright-seated (*B*) postures; peak change in LVC from baseline (ΔLVC_{peak}, ml/min/mmHg) in the supine (*C*) and upright-seated (*D*) postures. Young sedentary n = 12, old sedentary n = 12, old active n = 10, old endurance trained n = 10. * $p < 0.05$, significantly different from the supine posture. # $p < 0.05$, significantly different from the young; § $p < 0.05$, significantly different from the old sedentary. Values are mean ± SEM.

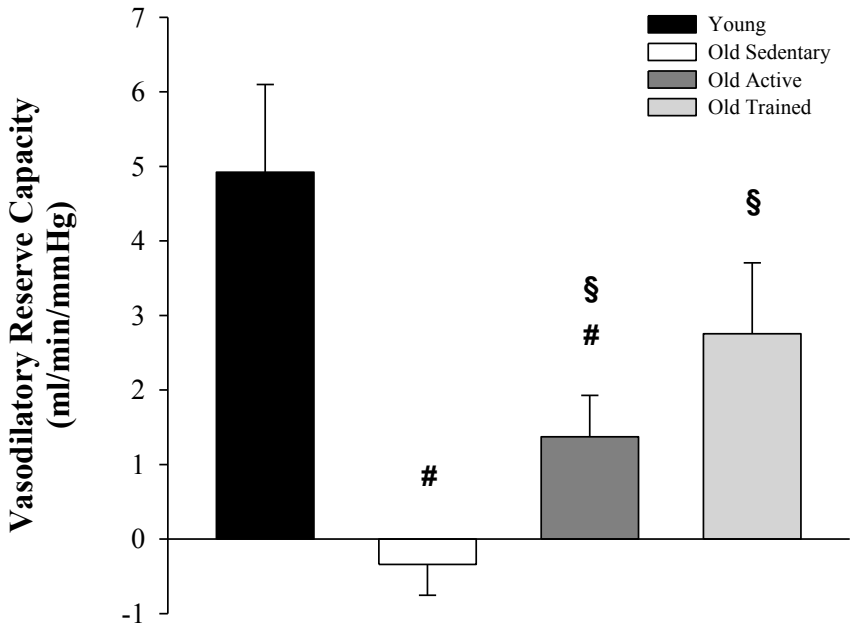

Figure 3.3 Passive leg movement (PLM)-induced vasodilatory reserve capacity with age and across physical activity and fitness levels. The difference in the PLM-induced peak change in leg vascular conductance (ΔLVC_{peak}, ml/min/mmHg) between the supine and upright-seated posture (vasodilatory reserve capacity). Young sedentary n = 12, old sedentary n = 12, old active n = 10, old endurance trained n = 10. # $p < 0.05$, significantly different from the young; § $p < 0.05$, significantly different from the old sedentary. Values are mean ± SEM.

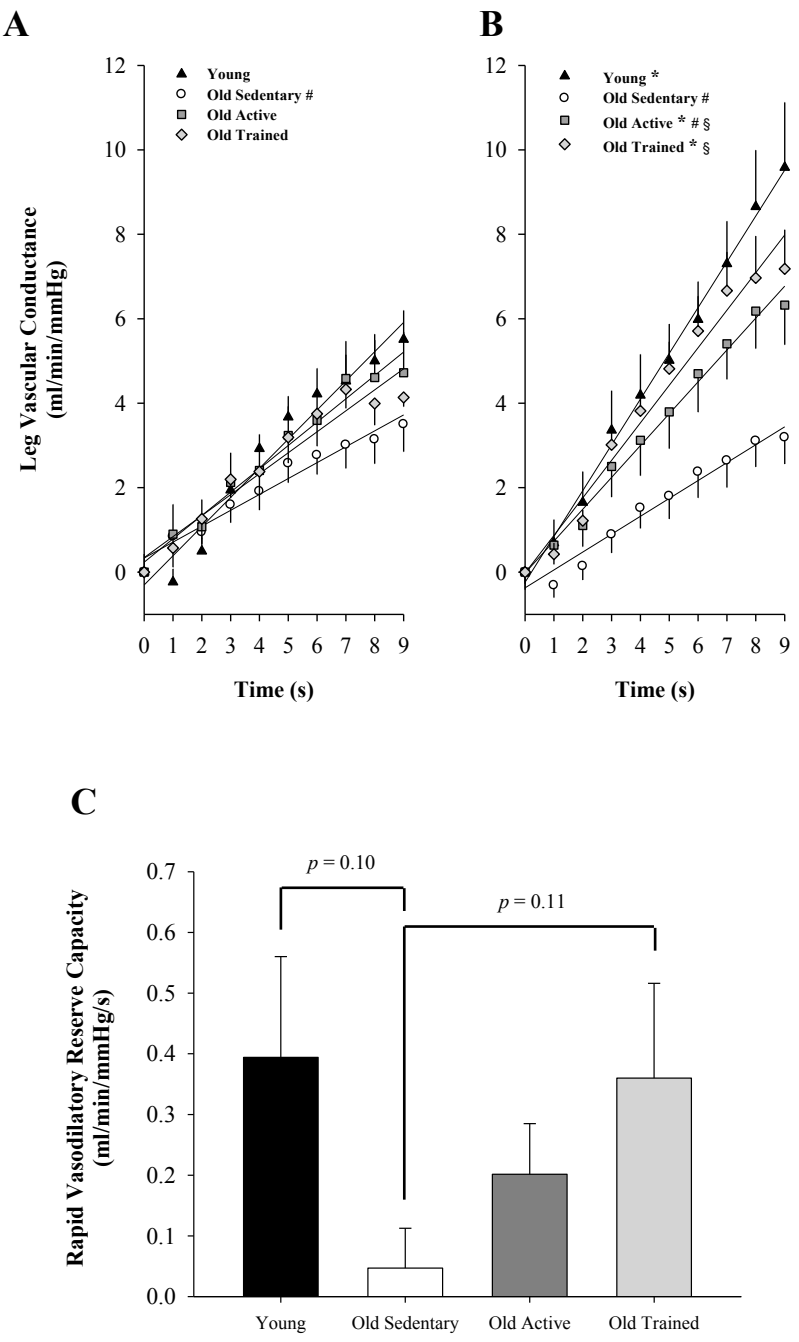

Figure 3.4 Passive leg movement (PLM)-induced rapid vasodilation in the supine and upright-seated posture with age and across physical activity and fitness levels. The rate of increase in the leg vascular conductance (LVC, ml/min/mmHg) over time was determined for the first 9 s of PLM in the supine (*A*) and upright-seated (*B*) postures; *C*, the difference in the slope between the supine and upright-seated posture (rapid vasodilatory reserve capacity, ml/min/mmHg/s). Young sedentary n = 12, old sedentary n = 12, old active n = 10, old endurance trained n = 10. * $p < 0.05$, significantly different from the supine posture. # $p < 0.05$, significantly different from the young; § $p < 0.05$, significantly different from the old sedentary. Values are mean ± SEM.

CHAPTER 4

DECREASED PASSIVE LEG MOVEMENT-INDUCED VASODILATION WITH AGE: THE IMPACT OF ACUTE ANTIOXIDANT SUPPLEMENTATION

Abstract

Passive leg movement (PLM)-induced vasodilation, a noninvasive measure of nitric oxide (NO)-mediated vasodilation, whether performed supine or upright to augment the contribution of NO, is attenuated with age. To examine the role of oxidative stress, supine and upright-seated PLM was performed in 15 young (22±1 yrs) and 17 old (72±2 yrs) healthy subjects after ingestion of an antioxidant cocktail (AOC, Vitamins C, E, and alpha-lipoic acid) or placebo. Hemodynamics were measured utilizing ultrasound Doppler and finger photoplethysmography. With placebo, PLM-induced peak change in leg vascular conductance (ΔLVC_{peak}) was significantly attenuated with age in both the supine (young: 6.6±0.7, old: 3.8±0.5 ml/min/mmHg) and upright-seated (young: 10.7±1.2, old: 3.7±0.7 ml/min/mmHg) posture. Although the old subjects had greater inflammation than the young (plasma Interleukin 6 (~ 335%) and C-reactive protein (~ 180%)), lipid peroxidation (malanaldehyde, MDA) was similar while plasma protein carbonyl levels tended to be higher in the old (~25%) compared to the young (p = 0.08). The AOC significantly improved antioxidant status in the young and old (plasma ascorbate and ferric reducing ability of plasma (FRAP)), and significantly attenuated lipid peroxidation (MDA). However, the AOC had no effect on the vasodilatory response to PLM in either group or posture (young supine: 7.1±1.0, young upright-seated: 10.8±1.4; old supine: 3.9±0.6, old upright-seated: 4.2±0.8 ml/min/mmHg). Therefore, vascular dysfunction persists in older adults despite an AOC-induced increase in antioxidant status and attenuated oxidative stress, questioning the direct role of redox balance in the apparent age-related decrease in NO bioavailability in the lower limbs.

Introduction

The impact of aging on cardiovascular disease (CVD) progression depends, to some extent, upon the health and function of the endothelium and, specifically, the bioavailability of the endothelium-derived vasodilator NO (23). NO is recognized to be antiatherogenic (25, 31), and NO bioavailability appears to fall with age (1, 9, 39). While the mechanisms leading to this age-related decline in NO bioavailability are not fully understood, one possible explanation is increased levels of free radicals, such as superoxide (O_2^-), resulting in NO scavenging and oxidative stress.

With age, pro-oxidant markers have been recognized to increase both in endothelial cells and circulating plasma and are associated with diminished endothelium-dependent vasodilation (7, 44), while the infusion of the antioxidant ascorbic acid in such subjects improves NO bioavailability and vasodilation assessed in the arm (10, 38, 39). Utilizing an AOC, our group has also demonstrated that lowering plasma markers of oxidative stress enhances endothelial function in the brachial artery of old subjects, as assessed by flow mediated vasodilation (FMD) (44). Thus, the literature provides compelling evidence that elevated free radical levels contribute to the attenuated vascular function observed in the arm with advancing age. However, somewhat surprisingly, to our knowledge, there is not a single study that has examined the role of oxidative stress on endothelium-dependent vasodilation in the lower limbs with age, where peripheral vascular disease is most common.

PLM, a novel tool for assessing vascular function, has recently been documented to reflect NO-mediated vasodilation in the lower limbs of young men (28, 41). In old subjects, the PLM-induced vasodilation is blunted compared to the young, primarily due

to a reduction in NO bioavailability (40). An additional feature of PLM is the opportunity to use posture (supine to upright-seated) to increase femoral perfusion pressure (FPP) (15) and potentially augment the role of NO in the vasodilatory response (14, 15). Indeed, in the young, this maneuver amplifies the rapid (first 9 s) vasodilatory response as well as the peak vasodilatory response to PLM, and reveals an NO-dependent vasodilatory reserve capacity (the difference in leg vascular conductance peak change (ΔLVC_{peak}) from supine to upright-seated posture). However, likely due to limited NO bioavailability, the old are unable to take advantage of this additional FPP-driven stimulus to vasodilate (14, 15). The underlying mechanism for this diminished NO-mediated vasodilation during PLM in the old is currently unknown, but could be related to the elevated oxidative stress typically associated with aging.

Consequently, utilizing PLM in both the supine and upright-seated posture to assess vascular function, we sought to determine the impact of acute AOC supplementation on the vasodilatory response in young and old subjects. We hypothesized that, due to a more pro-oxidant state and subsequently increased NO scavenging in the old, the AOC would 1) rescue the decreased PLM-induced vasodilation in the old in both body postures, 2) restore the NO-mediated component of the rapid vasodilatory response and the vasodilatory reserve capacity in the old, and 3) have no such hemodynamic effects in the young due to the more appropriate redox balance in this population.

Methods

Subjects

32 healthy subjects (15 young, 17 old) participated in this study. Subjects were included based on an absence of overt cardiovascular or metabolic disease, and aged 18-25 yrs for the young, and greater than 65 yrs for the old. Subjects refrained from taking any vitamin supplements for 7 days prior to enrolment in the study, and throughout the study period. All procedures were approved by the Institutional Review Boards of the University of Utah and the Salt Lake City VA Medical Center, and written informed consent was obtained from each participant prior to inclusion in the study. The study conformed to the standards set by the Declaration of Helsinki.

Experimental Protocol

All subjects participated in a familiarization trial and two experimental trials, each performed on separate days. During the familiarization trial, instrumentation followed by PLM was performed to both acquaint subjects with the experimental procedures and to ensure their ability to remain relaxed throughout the protocol. Prior to arrival for the experimental trials, subjects received either the AOC or placebo (PL) in a counterbalanced, single-blind, cross-over design.

Subjects reported to the experimental trials fasted and having refrained from caffeine for 12 and 24 hours prior, respectively. All young females were studied within the first 7 days (follicular phase) of the menstrual cycle to control for variations in circulating hormones, and all older females were self-confirmed postmenopausal. Blood samples were obtained from the antecubital vein to assess metabolic and blood cell parameters, as well as markers of oxidant and antioxidant status. Subjects were then

randomly assigned to begin in either the supine or upright-seated posture, instrumented, and allowed to rest for at least 20 min prior to initiation of the PLM protocol. Central and peripheral hemodynamic measurements were then collected for 1 min of baseline with the experimental leg supported at 180° knee joint angle, followed by 1 min of PLM through a 90° range of motion at a rate of 1 Hz. The contralateral leg remained motionless and supported at 180° knee joint angle throughout the protocol. PLM was performed by a member of the research team with real-time feedback provided by a position sensor and digital display to ensure full range of motion. A metronome, initiated prior to the start of baseline data collection, was used to maintain cadence. Prior to the start and throughout the protocol, subjects were encouraged to remain passive and resist the urge to help or hinder the movement. The PLM protocol was repeated in the opposing body posture (supine of upright-seated) after a rest period of at least 20 min in the new posture. These procedures were repeated on the second experimental day under the opposing supplement condition (AOC or PL).

Antioxidant Supplementation

Prior to arrival for the experimental trials, subjects received either AOC or PL in a counterbalanced, single-blind, crossover design. The AOC was administered in 2 doses, separated by 30 min, to improve absorption and maintain the time of antioxidant efficacy. The first dose was taken 90 min prior to the PLM protocol and consisted of 500 mg of Vitamin C, 200 IU of Vitamin E, and 300 mg of alpha-lipoic acid. The second dose, taken 60 min prior to PLM, consisted of 500 mg of Vitamin C, 400 IU of Vitamin E, and 300 mg of alpha-lipoic acid. Previously, this AOC and the dosing protocol, has been documented to lower the abundance of oxygen centered free radicals, as measured by

electron paramagnetic resonance spectroscopy, and improve vascular function (33). The PL consisted of microcrystalline cellulose capsules of similar taste and appearance, administered in 2 doses with identical time frame to the AOC.

Measurements

Central hemodynamic variables. Heart rate (HR) was determined using an electrocardiogram (ECG), while mean arterial pressure (MAP), stroke volume (SV), and cardiac output (CO) were assessed using finger photoplethysmography with a finometer (Finapres Medical Systems, Amsterdam, The Netherlands) positioned at heart level. SV was calculated using the model flow method (Beatscope, version 1.1; Finapres Medical Systems), and CO was calculated as the product of SV and HR.

Peripheral hemodynamic variables. Measurements of blood velocity and vessel diameter were performed in the common femoral artery of the passively moved leg, distal to the inguinal ligament and proximal to the bifurcation of the superficial and deep femoral artery using a Logic 7 ultrasound system (General Electric Medical Systems, Milwaukee, WI, USA). The Logic 7 was equipped with a linear array transducer operating at an imaging frequency of 14 MHz. Common femoral artery diameter was determined at a perpendicular angle along the central axis of the scanned area, and blood velocity was measured using the same transducer with a frequency of 5 MHz. All blood velocity measurements were obtained with the probe appropriately positioned to maintain an insonation angle of 60° or less. The sample volume was maximized according to vessel size and was centered within the vessel based on real-time ultrasound visualization. Using common femoral artery diameter and mean velocity (V_{mean}) (angle corrected, and intensity weighted), leg blood flow was automatically calculated by

commercially available software (Logic 7) as $V_{mean}\pi(\text{vessel diameter}/2)^2 \times 60$, where blood flow is in milliliters per minute. LVC was calculated as leg blood flow divided by MAP, and ΔLVC_{peak} was calculated as peak LVC minus baseline LVC.

Blood assays. Plasma and serum samples were stored at -80° until analysis. Both plasma IL6 and CRP (R&D Systems, Inc., Minneapolis, MN), indicators of systemic inflammation, were only measured under PL conditions, as they would not be expected to change with acute AOC supplementation. The efficacy of the AOC supplementation protocol, in terms of Vitamin C delivery, was determined from plasma ascorbate concentration (CosmoBio, Carlsbad, CA) and total antioxidant capacity was assessed by the ferric reducing ability of plasma (FRAP) assay, using the method of Benzie and Strain (3). Enzymatic antioxidant activity, assessed by superoxide dismutase (SOD), was also assayed in the plasma (43) (Cayman Chemical Company, Ann Arbor, MI). Lipid peroxidation was assessed by plasma malondialdehyde levels (MDA, Bioxytech LPO-586, Foster City, CA), while protein oxidation was evaluated by plasma protein carbonyl levels (Bloodworks Northwest, Seattle, WA). Glucose, lipids, and a complete blood cell count were performed using standard techniques.

Knee joint angle. During each protocol, knee joint angle of the passively moved leg was continuously recorded using a Vishay Spectrol 360 degree Smart Position Sensor (Vishay Intertechnology Inc., Malvern, PA, USA) mounted on a BREG X2K knee brace (BREG Inc., Vista, CA, USA) worn by the participants.

Anthropometrics and physical activity. Body mass and height were recorded and used to calculate body mass index (BMI) as BMI = body mass x height^2, where body mass is measured in kilograms and height is measured in meters. Thigh volume of the

passively moved leg was calculated, as previously described (24), using three measurements of thigh circumference (proximal, middle, and distal), thigh length, and skinfold measurements. Physical activity level was assessed using accelerometry and expressed as both total activity counts per minute and total steps per day.

Data Acquisition and Statistical Analysis

Throughout each protocol ECG, SV, CO, MAP, and knee joint angle signals underwent analog-to-digital conversion and were simultaneously acquired (200 Hz) using commercially available data acquisition software (AcqKnowledge, Biopac Systems Inc., Goleta, CA, USA). Common femoral artery diameter and V_{mean} were acquired with the ultrasound system (GE Logic 7). Baseline was analyzed using the average of the 60 s prior to the initiation of PLM. All variables were analyzed second-by-second for the 60 s of PLM, and data were smoothed using a 3 s rolling average prior to final data analysis. 2x2x2 repeated measures ANOVA were used to determine significant differences at baseline and in the absolute change from baseline to peak for HR, SV, CO, MAP, LBF and LVC, as well as for the slope of LVC across time for the first 9 s of PLM. A 2x2 repeated measures ANOVA was used to compare the vasodilatory reserve capacity between groups and conditions, and student's t-tests were utilized for subject characteristics. Significance was set at an α-level of 0.05, and all data are presented as mean ± SEM.

Results

Subjects

Subject characteristics are displayed in Table 4.1. Body mass and BMI were significantly higher in the older group compared to the young, but thigh volume was not different. Fasting blood glucose and total cholesterol were significantly higher in the older group, but glucose levels remained within the recommended range. Hemoglobin was similar between groups, as was the complete blood cell count, with the exception of Lymphocytes, which were lower in the old. Both IL6 and CRP levels were significantly elevated in the older group. Total physical activity, expressed as both activity counts per minute and steps per day, tended to be lower in the old, but this did not reach statistical significance ($p = 0.08$ and $p = 0.09$, respectively).

Oxidant/Antioxidant Status

Plasma Vitamin C, FRAP, and MDA levels are displayed in Figure 4.1. Vitamin C levels increased significantly with the AOC in both the young and the old (~61% and ~48%, respectively, $p < 0.05$), with no difference between groups during either the PL or AOC condition. Additionally, FRAP was significantly increased with the AOC (young: ~4%; old: ~7%; $p < 0.05$), and MDA was significantly decreased with the AOC (young: ~6%; old: ~8%, $p < 0.05$), but neither FRAP nor MDA were different between groups during either the PL or the AOC condition. Plasma protein carbonyl levels tended to be higher in the old (~25%) compared to the young in the PL condition, but this did not reach statistical significance (young: 0.1 ± 0.005; old: 0.124 ± 0.009 η/ml; $p = 0.08$). The AOC did not have a significant effect on protein carbonyl levels in either the young or the old, but the tendency for higher levels in the old was no longer evident (young: 0.105 ±

0.01; old: 0.115 ± 0.008 η/ml), $p = 0.55$). Plasma SOD activity was not different between the young and the old in the PL condition (young: 13.7 ± 2.4; old: 13.6 ± 0.05 IU/ml, $p = 0.88$), and, although tending to increase with the AOC in the old (~5%, $p = 0.09$), remained undifferentiated compared to the young (young: 14.3 ± 0.08; old: 14.9 ± 0.7 IU/ml), $p = 0.65$).

Central Hemodynamics at Rest and in Responses to PLM

Central hemodynamic variables are displayed in Table 4.2. During resting baseline, MAP was significantly greater in the old compared to the young during all conditions except the upright-seated AOC condition, which tended to be elevated with age ($P = 0.06$). There were no age, posture, or condition effects evident during resting baseline for CO, SV, or HR. During PLM in the old, the ΔMAP_{peak} during both the PL and AOC condition in the supine posture decreased to a greater extent compared to the young, with no age-related difference in ΔMAP_{peak} in the upright-seated posture. There were no differences between age groups in ΔCO_{peak}, ΔSV_{peak}, or ΔHR_{peak} responses to PLM for either body posture or condition.

Peripheral Hemodynamics at Rest and in Responses to PLM

Peripheral hemodynamic variables are displayed in Table 4.2. During resting baseline, LBF and LVC were attenuated in both the PL and AOC conditions in both body postures. In the young, LBF and LVC were elevated during the AOC condition in the supine posture, and LVC was significantly lower in the upright-seated compared to supine posture during the AOC condition only. In the old, the AOC had no effect on resting LBF or LVC. PLM resulted in a significant increase in LBF and LVC in both age

groups and across all trials. In the supine posture, the PLM-induced ΔLBF_{peak} and ΔLVC_{peak} were attenuated with age during both the PL (ΔLBF_{peak}: ~40%, P=0.07; ΔLVC_{peak}: ~42%, $p < 0.05$) and the AOC condition (ΔLBF_{peak}: ~45%, P<0.05; ΔLVC_{peak}: ~45%, $p = 0.06$; Table 4.2, Figure 4.2A and B). In the young, the upright-seated posture significantly augmented the ΔLBF_{peak} and ΔLVC_{peak} during both PL and AOC conditions ($p < 0.05$), with no change in either the PL or AOC condition in the old (Table 4.2, Figure 4.2C & D). Therefore, the ΔLBF_{peak} and ΔLVC_{peak} in the upright-seated posture were significantly attenuated in the old compared to the young during both the PL (ΔLBF_{peak}: ~59%, P<0.05; ΔLVC_{peak}: ~65%, $p < 0.05$) and the AOC condition (ΔLBF_{peak}: ~59%, P<0.05; ΔLVC_{peak}: ~61%, $p < 0.05$). Additionally, the AOC had no effect on the PLM-induced hyperemic response in either the young or old group or in the two body postures.

The slope of LVC over time for the first 9 s was significantly attenuated with age under PL conditions in both the supine (young: 0.63 ± 0.10 ml/min/mmHg; old: 0.28 ± 0.06 ml/min/mmHg; $p < 0.05$; Figure 4.3A) and upright-seated postures (young: 0.98 ± 0.12 ml/min/mmHg; old: 0.32 ± 0.07 ml/min/mmHg; p<0.05; Figure 4.3B), as well as during the AOC condition (young supine: 0.68 ± 0.11 ml/min/mmHg; old supine: 0.28 ± 0.05 ml/min/mmHg; young upright-seated: 1.09 ± 0.14 ml/min/mmHg; old upright-seated: 0.34 ± 0.078 ml/min/mmHg; $p < 0.05$ between groups for a given body posture; Figure 4.3A and B). In the young, the upright-seated posture increased the PLM-induced rapid vasodilatory response during both the PL and AOC conditions ($p < 0.05$), with no change in the old. However, AOC supplementation had no effect on the rapid vasodilatory response to PLM in either the young and old group or in the two body

postures.

The young exhibited a significant vasodilatory reserve capacity in both the PL and AOC conditions (Figure 4.4). However, the AOC had no effect on the magnitude of the vasodilatory reserve (PL: 4.1 ± 0.9 ml/min/mmHg, AOC: 3.8 ± 1.0 ml/min/mmHg, p = 0.77). The old lacked a vasodilatory reserve capacity in the PL conditions and the AOC was unable to augment it (PL: 0.0 ± 0.5 ml/min/mmHg, AOC: 0.3 ± 0.7 ml/min/mmHg, p = 0.53), resulting in a significantly attenuated vasodilatory reserve capacity under both conditions compared to the young ($p < 0.05$).

Discussion

Utilizing the PLM model, a noninvasive measure of NO-mediated vasodilation, and the acute administration of an AOC, this study sought to determine the role of oxidative stress in the age-associated attenuation of lower limb vascular function. In agreement with previous findings, the hyperemic response to PLM was attenuated in the old, who remained unresponsive to the upright-seated posture, as evidenced by the absence of a vasodilatory reserve capacity. Although the old subjects exhibited greater inflammation than the young, lipid peroxidation was similar while plasma protein carbonyl levels tended to be higher in the old compared to the young. The AOC significantly improved antioxidant status in the young and old, significantly attenuating lipid peroxidation and ameliorating the tendency for higher protein oxidation in the old. However, the AOC had no effect on the vasodilatory response to PLM in either group or posture. Furthermore, both the vasodilatory reserve capacity and the rapid vasodilatory response in the upright-seated posture, previously documented to reflect additional markers of NO-mediated vasodilation, remained suppressed in the old subjects.

Therefore, vascular dysfunction persists in older adults despite an AOC-induced increase in antioxidant status and attenuated oxidative stress, questioning the role of redox balance in the apparent age-related decrease in NO bioavailability in the lower limbs.

Free Radicals, Antioxidants, and Redox Balance

In typical healthy conditions, intravascular cell free radical production is opposed by enzymatic and nonenzymatic antioxidant defenses, limiting the impact of free radically-mediated oxidizing reactions. Indeed, in young healthy individuals, this balance between pro- and antioxidant forces allows oxidants to act as intracellular signaling molecules while preventing oxidant levels from increasing to a point that could cause significant oxidative stress and cellular damage (6, 13, 20, 35, 37). Perhaps initiated, or at least perpetuated, by inflammation, when this redox balance is tipped in favor of pro-oxidant forces, damage to proteins, DNA, and second messengers (i.e., oxidative stress) may lead to cellular dysfunction and/or apoptosis (21, 37). One such molecule is O_2^-, a free radical produced in vascular cells by the activation of NAD(P)H oxidase, xanthine oxidase, mitochondrial respiration, and uncoupled endothelial nitric oxide synthase (eNOS) (6, 37). O_2^- reacts rapidly with NO to form peroxynitrite ($ONOO^-$), decreasing NO bioavailability and impairing vasodilation (26, 37, 42). Therefore, in individuals with elevated oxidative stress, decreasing free radicals may improve vascular function.

Delivery of an AOC, as utilized in this study, including Vitamins C and E, and alpha-lipoic acid, should assist in counteracting the negative consequences of excess free radicals and sustain a healthy redox balance. Vitamin C scavenges free radicals, such as O_2^- and the hydroxyl radical (OH), by donating an electron and proton to form a reduced, nonradical product (12, 37). Decreasing the concentration of free radicals has been

demonstrated to limit the oxidation of Tetrahydrobiopterin (BH_4), an important eNOS cofactor, thereby decreasing eNOS uncoupling and leading to greater NO bioavailability (2, 5, 26, 37). Additionally, Vitamin C inhibits LDL lipid peroxidation by vascular cells by scavenging protein radicals that initiate this process (4, 37). Vitamin E is a lipid soluble antioxidant that tends to localize to lipoproteins and cell membranes. As such, Vitamin E is the major antioxidant scavenger of lipid-derived peroxyl radicals, leading to the formation of a product that is nearly nonreactant (36, 37). In addition to attenuating lipid peroxidation, Vitamin E can also scavenge $ONOO^-$ to form nonradical products, the combination of which acutely decreases the concentration of free radicals (36, 37). Finally, alpha-lipoic acid acts both as a direct antioxidant, by quenching hydroxyl and hypochlorous radicals, and also in the chelation of redox active metals (30, 34). Additionally, alpha-lipoic acid has been documented to decrease O_2^- levels and improve NO-mediated vasodilation, likely achieved through its reduced form, dihydrolipoic acid (16, 30, 34). In the current study, the combination of Vitamin C and E and alpha-lipoic acid in the AOC significantly improved antioxidant status in both the young and old (Figure 4.1), significantly attenuated lipid peroxidation in both young and old, and ameliorated the tendency for higher protein oxidation in the old.

Oxidative Stress and Vascular Function with Age

Previous research has demonstrated that vascular function is attenuated in the old compared to the young, and that this decrement can be attributed to oxidative stress. Intra-arterial infusion of Vitamin C into the brachial artery of older humans improves endothelium-dependent vasodilation, whether induced by increasing wall shear stress (10, 19) or by acetylcholine infusion (38, 39), with no effect in young subjects. However,

these studies did not directly measure the oxidant/antioxidant status of the subjects, and rather assumed that the older individuals possessed vascular oxidative stress, given that antioxidant supplementation improved their vascular function. In a study utilizing the same oral AOC supplementation as the current investigation, Wray et al. (2012) measured blood markers of oxidant/antioxidant status, finding that serum thiobarbituric acid reactive substances (TBARS), a measure of blood born oxidants similar to MDA, were elevated at baseline in the old compared to the young (44). Interestingly, the old also demonstrated elevated FRAP compared to the young, perhaps suggesting that in this group, antioxidants were increased in response to elevated oxidants. Despite this attempt to "rebalance" pro and antioxidant forces, endothelium-dependent vasodilation in the brachial artery, assessed by FMD, was attenuated in the old compared to the young, and rescued after AOC supplementation. Finally, Donato et al. (2007) measured both brachial artery FMD and markers of oxidative stress and antioxidant capacity from arterial and venous endothelial cells harvested from the same subjects (7). The results revealed that older subjects had decreased endothelium-dependent vasodilation that was inversely related to levels of nitrotyrosine, a marker of protein oxidation. NAD(P)H oxidase was also elevated in the venous endothelial cells of the old men, while, similar to the current study, there was no evidence of an increase in endogenous antioxidants between the young and old groups (7). In combination with the current data (Figures 1-4), these findings reveal highly variable findings in terms of pro- and antioxidant levels with age, but consistent evidence of vascular dysfunction, which, until now, has predominantly been assessed in the upper limb.

Antioxidants and Vascular Function in the Leg with Age

Although the old subjects in this study had greater inflammation than the young (IL6 and CRP), lipid peroxidation (MDA) was similar, while evidence of protein oxidation (protein carbonyls) tended to be higher in the old compared to the young (Figure 4.1). Therefore, despite the preponderance of evidence indicating that advancing age is associated with oxidative stress, in the current group of healthy older men such a finding was not so clear-cut. However, as expected, the AOC significantly improved antioxidant status in the young and old, significantly attenuating lipid peroxidation, and ameliorating the tendency for higher protein oxidation in the old.

The hemodynamic findings from the current investigation agree with previous research demonstrating an attenuated PLM-induced vasodilation with age (14, 15, 28, 40). In fact, here the NO component of PLM-induced vasodilation has been assessed utilizing three analysis techniques: the magnitude of the ΔLVC_{peak} (Figure 4.2), the rapid vasodilatory response (Figure 4.3), and the vasodilatory reserve capacity (Figure 4.4) (14, 40). All of these techniques indicate that NO bioavailability is decreased with age as assessed by the PLM model. More specifically, in the PL condition; 1) the ΔLVC_{peak} in the supine posture was attenuated with age (Figure 4.2B); 2) augmenting FPP with the upright-seated posture increased the ΔLVC_{peak} in the young with no change in the old (Figure 4.2D), resulting in the absence of a vasodilatory reserve capacity with age (Figure 4.4); and 3) while the slope of LVC over time increased in the young during augmented FPP, the rapid vasodilatory response did not change in the old (Figure 4.3). Finally, of significant importance, none of these observations were impacted by acute AOC supplementation. These assessments all indicate that NO bioavailability is attenuated in

this group of older subjects, despite less than obvious evidence of oxidative stress and clear impact of the AOC, questioning the direct role of redox balance in the apparent age-related decrease in NO bioavailability in the lower limbs.

The list of possible alternative mechanisms responsible for the diminished vascular function in the older subjects includes decreased eNOS content and/or phosphorylation, eNOS uncoupling due to decreased BH4 levels, and eNOS inhibition resulting from augmented caveolin-1 binding at the endothelial cell plasma membrane, among others. Interestingly, previous research has documented that eNOS protein content is preserved with age, while eNOS phosphorylation tends to increase (8, 29), the combination of which would augment NO bioavailability unless there was another deficiency. However, eNOS protein content and phosphorylation is only part of the equation. In the absence of BH4, eNOS becomes uncoupled and produces O_2^- rather than NO, a process that is exacerbated by uncoupled eNOS phosphorylation (22, 32). While decreased BH4 levels have been associated with aging and vascular dysfunction (11, 17, 27), and it is possible that the older subjects in our study had decreased BH4 and increased eNOS uncoupling, any resulting increase in free radical production should have been quenched by the AOC, leading to an improvement in vascular function. As this was not the case, it is unlikely that eNOS uncoupling is responsible for the age-related attenuation in vascular function. Finally, in the inactive state eNOS binds to caveolin-1 on the endothelial cell plasma membrane, a bond that must be broken in order for eNOS to become activated (18, 22, 32). It is possible that the age-associated increase in caveolin-1 (18, 45) prevents eNOS from being released into the cytosol where it can be stimulated to produce NO. Certainly, additional research is warranted to determine the

mechanisms by which PLM-induced vasodilation is attenuated with age.

Conclusion

The current investigation corroborates earlier reports that PLM-induced vasodilation, an indication of NO bioavailability, is attenuated with age, and extends these previous findings by demonstrating that the attenuation of NO-mediated vasodilation in the lower limbs of old subjects may not be a direct consequence of altered redox balance. Therefore, further research is warranted to elucidate the mechanisms responsible for the clearly diminished vascular function of the lower limbs with age.

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Table 4.1 Subject characteristics

	Young	Old
n (% Female)	15 (40)	17 (47)
Age (y)	22 ± 1	72 ± 2#
Height (cm)	172 ± 4	169 ± 3
Weight (kg)	68 ± 5	79 ± 5#
BMI (kg/m^2)	23 ± 1	27 ± 1#
Thigh Vol (dL)	60 ± 7	54 ± 5
CFA diameter (cm)	0.83 ± 0.04	0.91 ± 0.05
Glucose (mg/dl)	71 ± 2	78 ± 3#
Cholesterol (mg/dl)	170 ± 14	204 ± 15#
Triglycerides (mg/dl)	96 ± 30	109 ± 15
HDL (mg/dl)	49 ± 4	54 ± 5
LDL (mg/dl)	106 ± 10	122 ± 10
Haemoglobin (g/dl)	14.9 ± 0.4	14.7 ± 0.3
WBC (K/ul)	5.4 ± 0.3	5.5 ± 0.5
Neutrophil (K/ul)	2.8 ± 0.2	3.1 ± 0.4
Lymphocyte (K/ul)	2.0 ± 0.2	1.6 ± 0.2#
Monocyte (K/ul)	0.49 ± 0.03	0.48 ± 0.05
CRP (ηg/ml)	469 ± 70	1314 ± 243#
IL-6 (pg/ml)	0.69 ± 0.06	3.00 ± 0.66#
Activity counts/min	163 ± 22	126 ± 16
Steps/day	6690 ± 863	5279 ± 634

BMI, body mass index; CFA diameter, common femoral artery diameter; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; WBC, white blood cell; CRP, C-reactive protein; IL-6, interleukin 6. #$p < 0.05$ compared to the young. Values are mean ± SEM.

Table 4.2 Central and peripheral hemodynamics

	Rest							
	Supine				Upright-seated			
	Young		Old		Young		Old	
	Placebo	AOC	Placebo	AOC	Placebo	AOC	Placebo	AOC
MAP (mmHg)	88 ± 2	88 ± 2	98 ± 4#	96 ± 3#	86 ± 1	85 ± 2	98 ± 4#	92 ± 3
CO (l/min)	5.1 ± 0.2	5.7 ± 0.3	5.3 ± 0.4	5.4 ± 0.3	5.4 ± 0.2	5.8 ± 0.2	5.1 ± 0.3	5.5 ± 0.4
SV (ml/beat)	90 ± 5	88 ± 5	94 ± 8	96 ± 7	87 ± 6	88 ± 5	90 ± 7	95 ± 7
HR (beat/min)	58 ± 3	64 ± 3	57 ± 2	57 ± 2	63 ± 3	67 ± 3	59 ± 2	59 ± 2
LBF (ml/min)	323 ± 22	411 ± 60*	256 ± 18#	229 ± 21#	321 ± 38	322 ± 44	228 ± 15#	228 ± 20#
LVC (ml/min/mmHg)	3.7 ± 0.2	4.6 ± 0.6*	2.7 ± 0.3#	2.4 ± 0.2#	3.7 ± 0.4	3.7 ± 0.5†	2.4 ± 0.2#	2.5 ± 0.2#
	Passive Leg Movement (Δpeak)							
	Supine				Upright-seated			
	Young		Old		Young		Old	
	Placebo	AOC	Placebo	AOC	Placebo	AOC	Placebo	AOC
ΔMAPpeak (mmHg)	-6 ± 1	-4 ± 1	-9 ± 2#	-10 ± 2#	-5 ± 1	-5 ± 2	-7 ± 1	-6 ± 1
ΔCOpeak (l/min)	1.1 ± 0.3	1.0 ± 0.1	1.4 ± 0.4	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	0.9 ± 0.3	0.8 ± 0.2
ΔSVpeak (ml/beat)	10 ± 3	8 ± 1	12 ± 4	11 ± 3	11 ± 2	9 ± 2	10 ± 2	9 ± 2
ΔHRpeak (beat/min)	11 ± 1	10 ± 1	10 ± 3	10 ± 3	9 ± 2	10 ± 2	8 ± 4	7 ± 3
ΔLBFpeak (ml/min)	565 ± 59	627 ± 89	345 ± 51#	342 ± 46#	871 ± 92†	893 ± 120†	355 ± 64#	363 ± 60#
ΔLVCpeak (ml/min/mmHg)	6.6 ± 0.7	7.1 ± 1.0	3.8 ± 0.5#	3.9 ± 0.6#	10.7 ± 1.2†	10.8 ± 1.4†	3.7 ± 0.7#	4.2 ± 0.8#

MAP, mean arterial pressure; CO, cardiac output; SV, stroke volume; HR, heart rate; LBF, leg blood flow; LVC, leg vascular conductance; Δ_{peak}, peak change from baseline. *$p < 0.05$ compared to placebo, #$p < 0.05$ compared to the young, †$p < 0.05$ compared to the supine posture. Values are mean ± SEM.

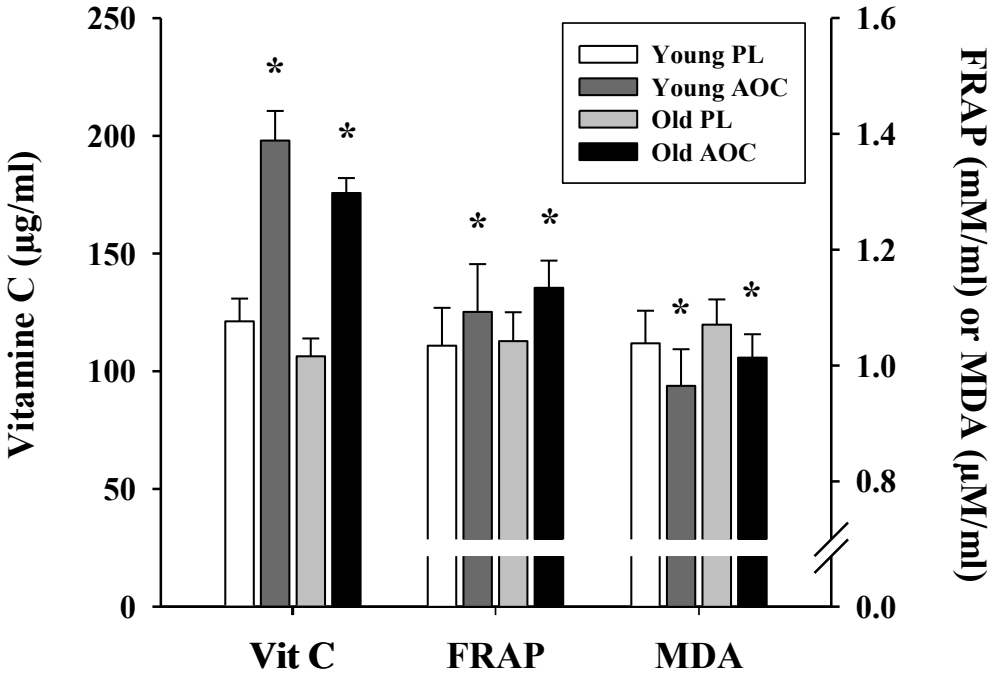

Figure 4.1 Blood markers of pro- and antioxidant status. The difference in pro- and antioxidant blood markers between groups and under placebo (PL) and antioxidant cocktail (AOC) conditions. The AOC significantly increased the antioxidant markers Vitamin C and the ferric reducing ability of plasma (FRAP), and decreased the oxidant marker malondialdehyde (MDA) in both young and old subjects compared to PL. Young n = 15, old n = 17. * $p < 0.05$ compared to PL. Values are mean ± SEM.

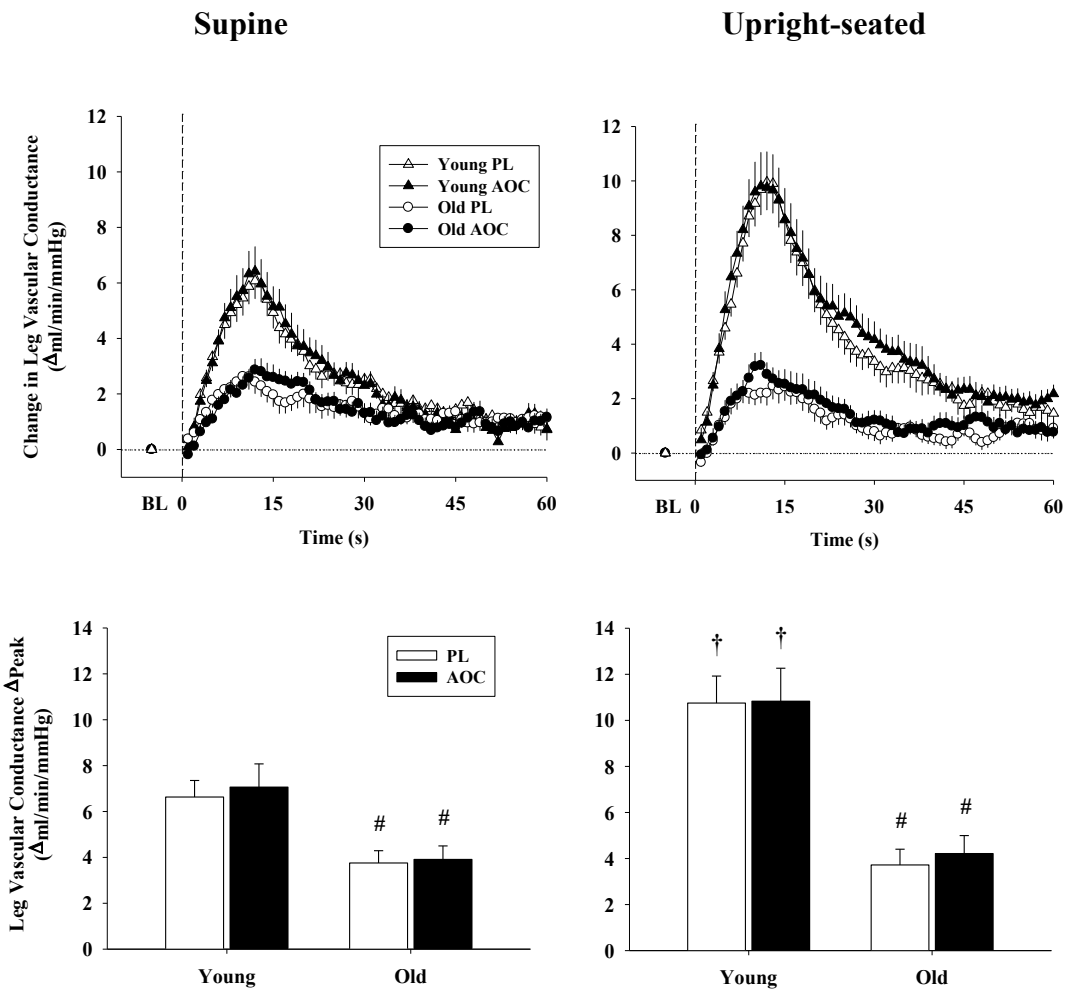

Figure 4.2 Passive leg movement (PLM)-induced change in leg vascular conductance. Second-by-second tracing of leg vascular conductance (LVC, ml/min/mmHg) at baseline (BL) and throughout 60 s of PLM in the supine (A) and upright-seated (C) postures under placebo (PL) and antioxidant cocktail (AOC) conditions. Dashed line at 0 s indicates the start of 60 s of PLM. The PLM-induced peak change in LVC from baseline (ΔLVC_{peak}, ml/min/mmHg) was significantly attenuated in the old compared to the young in the supine (B) and upright-seated (D) postures in both the PL and AOC conditions. Young n = 15, old n = 17. There was no difference between PL and AOC in either group or body posture. #$p < 0.05$ compared to the young; †$p < 0.05$ compared to supine posture. Values are mean ± SEM.

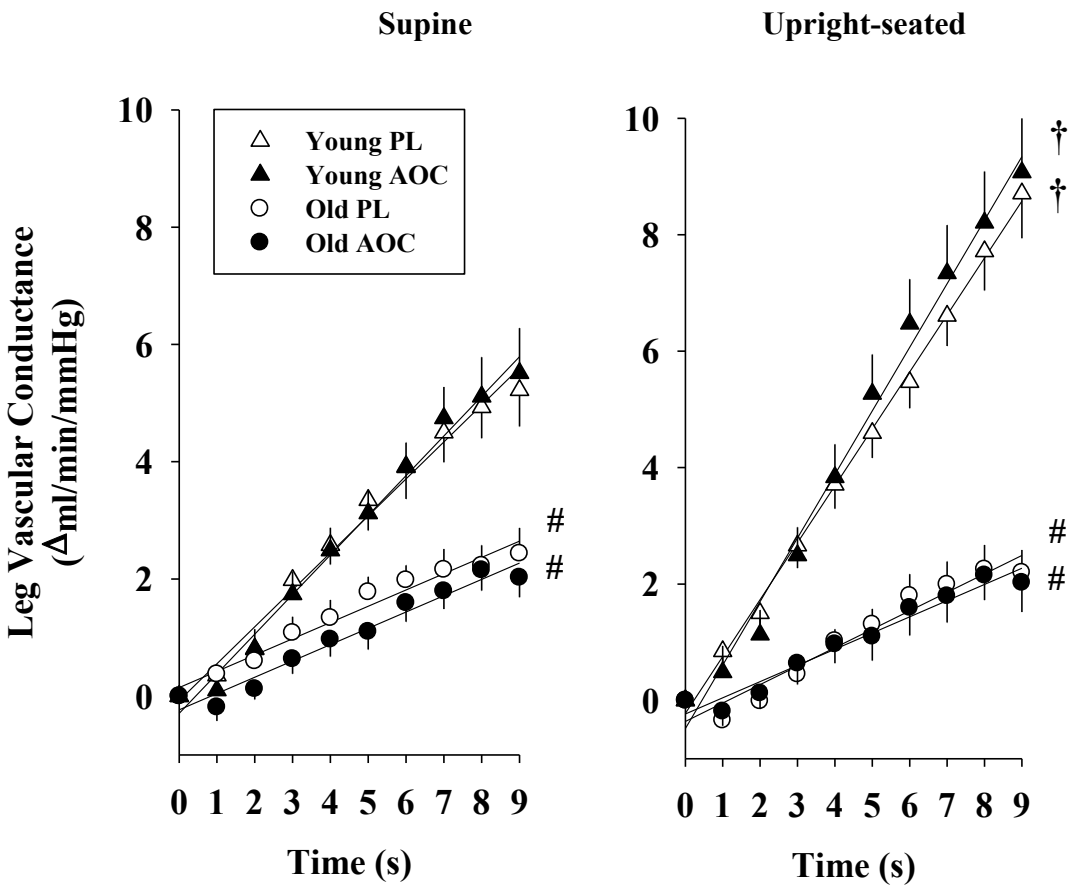

Figure 4.3 Passive leg movement (PLM)-induced rapid vasodilatory response. The slopes calculated for the increase in the leg vascular conductance (LVC, ml/min/mmHg) over time (9 sec) display an attenuated rapid vasodilatory response in the old in both the supine (A) and upright-seated (B) postures, with no effect of the antioxidant cocktail (PL = placebo, AOC = antioxidant cocktail). Young n = 15, old n = 17. There was no difference between PL and AOC in either group or body posture. #$p < 0.05$ compared to the young; †$p < 0.05$ compared to supine posture. Values are mean ± SEM.

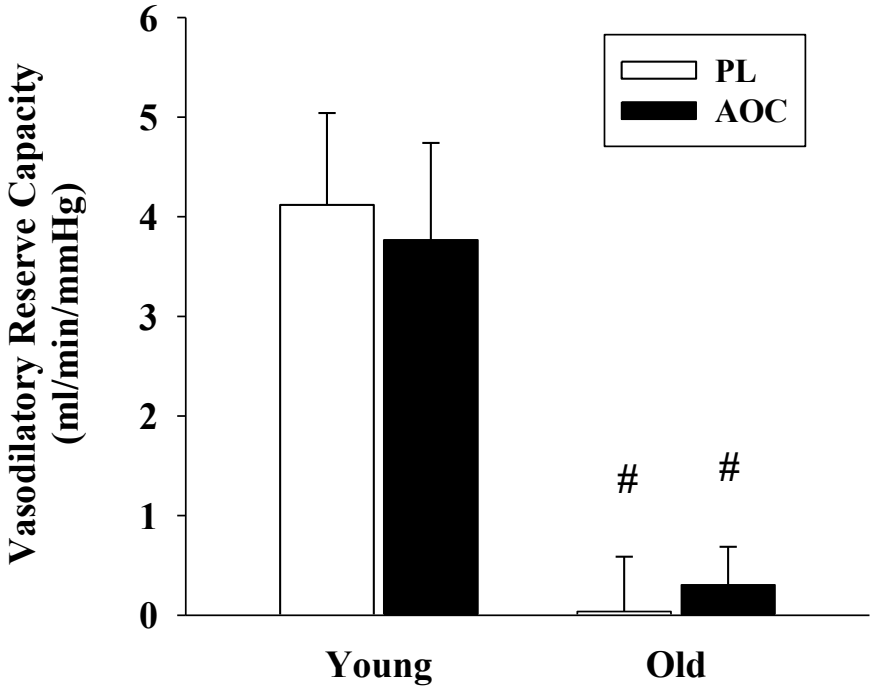

Figure 4.4 Passive leg movement (PLM)-induced vasodilatory reserve capacity. The difference in the PLM-induced peak change in leg vascular conductance (ΔLVC_{peak}, ml/min/mmHg) between the supine and upright-seated posture (vasodilatory reserve capacity) in the young (n = 15) compared to the old (n = 17) under both placebo (PL) and antioxidant cocktail (AOC) conditions. There was no effect of the AOC in either group. #$p < 0.05$ compared to the young. Values are mean ± SEM.

CHAPTER 5

CONCLUSION

The overall objective of this dissertation was to better understand alterations in vascular function with age in healthy humans by utilizing the PLM model, an assessment of predominantly NO-mediated vasodilation. Specifically, we examined the impact of aging on vascular function in women, the effect of greater physical activity and fitness levels on vascular aging, and the impact of acute antioxidant supplementation on vascular function with age. Collectively, by providing mechanistic insight, this research sought to identify strategies to improve vascular health, thereby lessening the adverse cardiovascular effects of human aging.

Results from the first study support previous findings that PLM-induced vasodilation is attenuated with age, and extend these observations to include women. Additionally, increasing FPP by moving from the supine to the upright-seated posture, which magnifies the role of NO in both the rapid and overall vasodilatory response, revealed a vasodilatory reserve capacity in young women, but not their old counterparts. These findings imply that NO bioavailability is attenuated with age in women, and add additional support to the utility of the PLM model as an assessment of NO-mediated vascular function across the human lifespan.

Study two again supports previous findings that aging has a deleterious effect on vascular function in otherwise healthy, sedentary humans, and adds to the literature by demonstrating that higher levels of physical activity and fitness can partially restore, or protect against, this age-associated decrease in PLM-induced vasodilation. The observation that the upright-seated ΔLVC_{peak}, PLM-induced vasodilatory reserve capacity, and rapid vasodilatory reserve capacity are predominantly NO dependent in the young suggests that the attenuated vascular function with age is due to decreased NO

bioavailability, which can be somewhat protected by increasing levels of physical activity and fitness with advancing age.

The third study in this dissertation demonstrated that despite increased antioxidant capacity in both young and old subjects after ingestion of an oral antioxidant cocktail, this improved redox balance did not lead to augmented vascular function in either group. Therefore, the decrease in NO-mediated vasodilation in the lower limbs of old subjects may not be a direct consequence of oxidative stress. Further research is warranted to elucidate the mechanisms responsible for the clearly diminished vascular function of the lower limbs with age.

Collectively, this research has expanded the literature by further characterizing age-associated declines in vascular function to include women and provided insight into the effects of physical activity and redox balance on the vasodilatory response with age. As decreased NO bioavailability and the subsequent attenuation in vascular function are linked to increased CVD risk and greater prevalence of morbidity and mortality, the methods developed and overall conclusions garnered from this dissertation have important implications for improving health and quality of life throughout human aging.